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Please add two new claims as follows:

46. (New) A method according to claim 38, wherein the *Plasmodium* species is selected from the group consisting of *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*.

47. (New) A method according to claim 38 wherein the agent results in the formation within the tissues of nitric oxide in the form of a compound of formula:

R-NO

Wherein R is an NO releasing, delivering or transferring moiety selected from the group consisting of an amino acid, peptide, polypeptide, protein, enzyme, amine, glycolipid, polysaccharide and a chemical derivative thereof.

REMARKS

Claims 38-47 are under prosecution in this case. Claims 38-45 have been amended for improved clarity. New claim 46 has been added to better claim the subject matter which Applicants regard as the invention. New claim 47 is a rewritten version of as-filed claim 43. No new matter has been added in this Amendment.

Claim Rejections under 35 U.S.C. §112:

Claims 38-45 are rejected under 35 U.S.C. §112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. Applicants respectfully traverse this rejection.

Without acquiescing to this aspect of the rejection, claims 38-45 have been amended such that the issues raised by the Examiner are no longer relevant. Specifically, the term, "NO modifying agent" has been deleted from claims 38, 43 and the antecedent support has been established for claim 40. Claim 44 has been canceled without prejudice. As-filed claim 43 has been rewritten in two claims (43 and 47). Claims 43 and 45 have been amended to recite proper Markush groups.

Applicants respectfully submit that claims 38-47 as submitted herein are clear and definite. Accordingly, withdrawal of the rejection under 35 U.S.C. §112, second paragraph, is respectfully requested.

Claim Rejections under 35 U.S.C. §103:

Claims 38-39, 42-43, and 45 are rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Seguin *et al.* in view of Green *et al.* Applicants respectfully traverse this rejection.

In contrast to the Examiner's allegation, Seguin *et al.* concern a mouse model of a disease that actually only exists in humans, namely, malaria. Furthermore, Seguin *et al.* used irradiated parasites in their mouse model to confer protective immunity. Use of irradiated parasites represents an artificial situation, used for the purposes of carrying out experiments in the mouse model system. Irradiated sporozoites do not behave *in vivo* as non-irradiated sporozoites would behave. Irradiated sporozoites persist *per se* in the liver. In contrast, non-irradiated sporozoites, which are ordinarily the causative agent in infection and disease in humans, replicate from one to thousands in the liver within about half an hour (the "liver stage") and then rupture out into the blood.

Because Seguin *et al.* used the artificial model of irradiated parasites, their paper only provides data on this artificial situation and comments only on the first stage of the life cycle of *Plasmodium*. This reference teaches nothing about the actual disease-causing stage, which occurs only once infecting organisms have multiplied and sporozoites have changed into merozoites which invade the red blood cells (the "blood stage"). That is, their use of sporozoites limits the relevance of the study to that of this first stage -- the liver stage -- and cannot be used to extrapolate to the second pathogenic stage -- the blood stage of the *Plasmodium* life cycle -- as no data concerning this stage are presented or discussed.

Moreover, the particular *Plasmodium* species used in Seguin *et al.* study -- *Plasmodium berghei* -- only infects mice; it never infects humans. Hence, it is

inappropriate and scientifically wrong to extrapolate the findings from the mouse study to the human *in vivo* situation.

Seguin *et al.*'s own statement, at the end of the Introduction, that their "results indicate that NO production is required for protection in irradiated sporozoite-immunized mice ..." indicates the very limitations of such experimentation. It is not possible to extrapolate their findings to protection from other stages of the *plasmodium* life cycle; to protection in any other cells other than liver cells; to protection against any other species other than *P. berghei*, or to protection in any other mammal other than a mouse.

The following highlights other key differences, well appreciated by a person of ordinary skill in the art, between a mouse model of malaria and the actual human *in vivo* disease:

1. In the mouse model of cerebral malaria using *Plasmodium berghei* (used by Seguin *et al.*), it is mouse white cells, not parasitized red cells, that cytoadhere (stick to) and clog up blood vessels. Parasitized red cells do not cytoadhere to the lining of blood vessels and do not clog up blood vessels. This is a fundamental difference which makes conclusions from this mouse model impossible to extrapolate to humans, as this cytoadherence and clogging process caused by parasitized *red* cells is a critically important way in which *Plasmodium falciparum* causes severe disease in humans.

2. There are no mouse models in which nitric oxide (NO) production is suppressed in severe malaria. In contrast, the present inventors provide such evidence in humans in the instant application. That is, the present application actually provides data -- and not just surmise and hypothesis -- to show that NO production *in vivo* is reduced (not increased) in severe malaria in humans.

3. The immune systems of mice are quite different from those of humans in many respects. Specifically, regarding NO/NO synthase, mouse monocytes and

macrophages are more capable of producing NO than human monocytes, and respond to different stimuli. This difference also makes it very difficult to extrapolate to humans, with any degree of certainty, results of studies in mice that link protection from malaria with NO production.

Regarding the Taylor Robinson mouse model, which uses *P. chabaudi*, and indicates that NO can kill parasites in blood *in vivo*, Applicants submit that no subsequent publication has been able to repeat these data, despite effort. Submitted herewith as Exhibits A and B are two articles (van der Heyde *et al.*, *The Journal of Immunology 165:* 165: 3317-3323, 2000, "Nitric Oxide is neither necessary nor sufficient for resolution of *Plasmodium chabaudi* malaria in mice"; Amante and Good *Parasite Immunol. 19(3):* 111-126, 1997) that demonstrate this problem.

With respect to Green *et al.* cited by the Examiner as the basis for rejecting the claims as obvious, Applicants provide the following response.

The Green patent claims "a method of killing or inhibiting proliferation of infectious or pathogenic microorganisms in a human or an animal", where the method is to cause the release of NO. First of all, this patent is directed only to affecting the actual parasitic microorganism, whatever that might be. It is not directed to prophylaxis or treatment of a disease; *i.e.*, it does not address the pathogenesis caused by the infection. Secondly, there are numerous examples of microorganisms that are not inhibited by NO: there is no suggestion in Green *et al.* that *Plasmodium* will be so affected. Thirdly, the cited patent is directed to causing release of NO, not to increasing production or delivery of NO. Furthermore, while NO may kill microorganisms *in vitro*, in the real world situation *in vivo* it often does not. For example, although NO prevents growth of *Borrelia burgdorferi* (the microorganism causing Lyme Disease) in *in vitro* culture, it does not kill or retard the growth of *Borrelia burgdorferi in vivo* (Seiler *et al.*, *Infect Immun. 63:* 3886, 1995). Similarly, there are mutants of *Salmonella* species that can evade killing by NO. Accordingly, Green *et al.* does not provide suggestion or

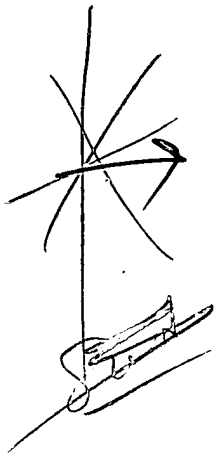
motivation for a person of ordinary skill in the art to use an agent that increases nitric oxide production to treat or prevent an infection by a *Plasmodium* species.

Green *et al.* is alleged to teach a method for treating infection by a pathogenic microbe, *Leishmania*, in humans and other animals. However, although *Leishmania* is a parasite that causes infection, it is quite distinct from malaria and *Plasmodium* infection. *Leishmania* never invades or infects red blood cells, as does *Plasmodium*. *Leishmania* lives within macrophages. The "NO releasing compound" of Green *et al.* induces cytotoxicity or cytostasis amongst macrophages to kill or inhibit the parasite. *Plasmodium* does not live within macrophages.

The "NO modifying agents", as referred to by the Examiner, are actually referred to by Green *et al.* as "NO releasing compounds" or "NO generator-containing compositions", and all are complexes of NO and another compound, specifically a nucleophile. These "generators" are designed to release NO from the complex.

Applicants emphasize that the teachings of Green would not have suggested or motivated a skilled artisan to use such a complex in the treatment of a completely different infection by a different parasite, which affects different cells and causes a different disease, in the hope that the release of NO by the complex would beneficially affect this other parasitic infection. This is particularly so given the observation that NO does not necessarily kill microorganisms *in vivo*. Certainly it is not obvious that such a "releasing compound" should advantageously affect disease progression following infection by this completely different organism. As already mentioned, Green *et al.* suggests nothing about inhibiting or retarding pathogenesis of the parasite; Green *et al.* is directed only to inhibiting or killing the parasitic organism. By contrast, the present invention is directed not only to inhibiting/killing *Plasmodium* (which are not in macrophages), but also to preventing the subsequent symptoms of the malaria disease.

Based on the foregoing, Applicants submit that the present invention cannot be regarded as *prima facie* obvious over Seguin *et al.* in view of Green *et al.* The data disclosed in Seguin *et al.* are not meaningful if extrapolated to human disease-stage malaria for the reasons described above. There is no suggestion in Green *et al.* that can motivate a person of ordinary skill in the art to combine the teachings therein with those of Seguin *et al.* to make the invention. The Examiner alleges that "... incorporate the teaching of Green, in administering by inhalation NO modifying agents that release NO, into the method of Seguin *et al.* that orally administers NO to induce NO production to kill intracellular *Plasmodium* species, *because Green specifically taught that directed delivery of NO into Leishmania infected macrophages kills the intracellular pathogen during the blood stage ...*" (emphasis added). The statement that "directed delivery of NO into *Leishmania* infected macrophages kills the intracellular pathogen during the blood stage" has no bearing whatever on the possible effect of NO on *Plasmodium*, which is never in macrophages.



Nonetheless, claim 38 has been amended to recite a non-mammal in order to advance the prosecution without acquiescing to the rejection. Applicants respectfully request that the rejection of claims 38-39, 42-43, and 45 under 35 U.S.C. §103 be withdrawn.

Claim 40 is rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Seguin *et al.* in view of Green *et al.* as applied to claims 38-39, 42-43, and 45, and further in view of Kremsner *et al.* Applicants respectfully traverse this rejection.

The shortcomings of the Seguin *et al.* and Green *et al.* references are discussed above. The shortcomings of the Kremsner *et al.* article have been discussed in the previous responses to the Examiner based on a refutation of the scientific reliability of the interpretation of the data of Kremsner *et al*, published in *Transactions of the Royal Society of Tropical Medicine and Hygeine 91:* 238-240, 1997.

The Examiner continues to assert that ".... Kremsner specifically taught that NO plays a protective role in the treatment of malaria infection by *P. falciparum* ...". However, nowhere in this paper are actual data presented to support the statement that clinical cure is associated with NO production.

Moreover, Kremsner *et al.* do not demonstrate or suggest a role for NO in protecting from the pathogenic processes of malaria. In particular, they do not discuss, indicate or suggest the ability of NO to down-regulate endothelial cell expression of ICAM-1 (deCaterina *et al., J Clin. Invest*, 1995), the major cytokine-inducible endothelial cell adhesion molecules involved in cytoadherence of parasitized red cells and microvascular sequestration/occlusion. This is a process central to the pathogenesis of severe malaria. Indeed, *Kremsner et al* instead quote Vidal *et al*'s 1992 findings, which are not subsequently supported by multiple 1993-96 publications available to Kremsner (e.g. DeCaterina *et al.*, 1995), and use this to caution that NO may enhance cytokine-induced cellular adhesion to endothelial cells. This is the opposite of the present invention, wherein NO ameliorates disease through preventing and/or reversing endothelial cytoadherence of parasitized red cells in malaria.

Furthermore, and most importantly, Kremsner *et al.* also claim that higher levels of NO metabolites are associated with severe disease and thus that increased NO production may be harmful in patients with malaria. They state that this "*supports the hypothesis that NO may have a causative role in malaria pathology*" (emphasis added). Again, this assertion is the direct opposite of that made in the instant application, and would teach a person of ordinary skill in the art away from making the invention. It reflects, in fact, the prevailing hypotheses at the time and contradicts their statement that NO predicts cure. There are similarly contradictory remarks throughout the paper that would confuse a person skilled in the art as to what the result would be if such a person were to try to use NO in treating malarial symptoms. It would not be clear whether to try to increase NO or to attempt to reduce it.

The claimed invention is a method of the prophylaxis and treatment of infection by the *Plasmodium* species by administering an agent that increases NO production or delivery. This invention was based on the actual data in humans generated by the inventors, which was the opposite of the view of the field at the time of the priority date. The prevailing view at the time was that NO is contra-indicated in malaria. This is perhaps best and most vehemently argued by Ian Clark in his exhaustive review article of 1996 (Clark and Rockett *Adv. Parasitol.* 37:1-56, 1996: *Adv. in Parasitology 37:* 1-56, 1996). Based on the conclusions of Kremsner and arguments of Clark, *inter alia*, a person of ordinary skill in the art would believe that NO mediates and contributes to pathology of severe malaria and would therefore not want to elevate NO in severe malaria. Indeed, they would be in favor of inhibiting NO.

Hence, given the confused messages contained both in the Kremsner paper and in the prevailing literature at the priority date and since, Kremsner *et al.* cannot be interpreted as prior art rendering the present invention obvious. The instant application is based on findings that NO production is lowest (not highest, as erroneously asserted by Kremsner *et al.*) in severe malaria.

In summary, based on the foregoing, the rejection based on the allegation that it would have been obvious to a person skilled in the art, at the priority date, to use an agent that increases NO production as a means of either preventing or treating *Plasmodium* infection and the subsequent pathological effects caused thereby is not sustainable. Withdrawal of the rejection under 35 U.S.C. §103(a) is respectfully requested.

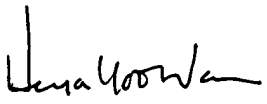
Conclusion:

Based on the foregoing amendments and arguments, this case is considered to be in condition for allowance and passage to issuance is respectfully requested.

If there are any outstanding issues related to patentability, the courtesy of a telephone interview is requested, and the Examiner is invited to call to arrange a mutually convenient time.

This Amendment is accompanied by a Petition for Extension of Time (three months) and a check in the amount of $465.00 as required under 37 C.F.R. §1.17(a)(1) for a small entity. If the amount submitted is incorrect, however, please charge any deficiency or credit any overpayment to Deposit Account No. 07-1969.

Respectfully submitted,



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In the Claims:

38. (Twice amended) A method for the prophylaxis or treatment of infection by a *Plasmodium* species in a non-rodent mammal, said method comprising administering to said non-rodent mammal [by inhalation an NO modifying agent which kills, inhibits or otherwise retards] an agent that increases nitric oxide production or delivery, thereby killing, inhibiting, or otherwise retarding the growth, infectivity or pathogenesis of the *Plasmodium* species.

39. (Once amended) A method according to claim 38 wherein the non-rodent mammal is a human.

40. (Once amended) A method according to claim [38] 46 wherein the [protozoa] *Plasmodium* species is *Plasmodium falciparum*.

41. (Twice amended) A method according to claim 38 wherein the agent is administered by inhalation to increase systemic nitric oxide levels or nitric oxide effect. [NO.]

42. (Once amended) A method according to claim 38 wherein the agent is a [NO] nitric oxide (NO) donor.

43. (Once amended) A method according to claim 38 wherein the [NO modifying] agent results in the formation within the circulatory system [and/or tissues of NO] of nitric oxide in the form of a compound of formula:

R-NO

Wherein R is an NO releasing, delivering or transferring moiety [such as] selected from the group consisting of an amino acid, peptide, polypeptide, protein, enzyme, amine, glycolipid, polysaccharide [or] and a chemical derivative thereof.

Please cancel claim 44 without prejudice.

45. (Once amended) A method according to claim 38 wherein the [NO modifying] agent [includes or is derived] is selected from the group consisting of cysteinylglycine, cysteine, cysteamine, lipoic acid, dithiothreitol, glutathione, L-arginine, penicillamine, N-acetyl-penicillamine, N-acetylcysteine, albumin, tissue plasminogen activator, streptokinase, a cytokine, [or] an antagonist or agonist of a cytokine [(*e.g.*, an antibody to a cytokine or soluble receptor for a cytokine or a fragment of a cytokine or a cytokine binding protein)], interferon $(IFN)_{\alpha}$, IFN_{β}, IFN_{γ}, [including IFN_{α}, IFN_{β}, IFN_{γ}, a growth factor including] granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), an interleukin (IL) [including IL] 1 to IL13, hemoglobin and cathepsin B.

Nitric Oxide Is Neither Necessary Nor Sufficient for Resolution of *Plasmodium chabaudi* Malaria in Mice[1]

Henri C. van der Heyde,[2*‡] Yang Gu,* Qiang Zhang,* Guang Sun,* and Matthew B. Grisham[†‡]

Malaria is a life-threatening re-emerging disease, yet it is still not clear how bloodstage malarial parasites are killed. Nitric oxide (NO), which has potent anti-microbial activity, may represent an important killing mechanism. The production of NO during descending *Plasmodium chabaudi* parasitemia, a period when parasites are killed by the immune response, supports this concept. However, NOS2$^{0/0}$ and NOS3$^{0/0}$ mice as well as mice treated with NO synthase 2 (NOS2) inhibitors do not develop exacerbated malaria, indicating that NO production is not necessary for the suppression of *P. chabaudi* parasitemia. It is possible due t the plasticity in the immune response during malaria that Ab-mediated immunity is enhanced in the absence of NO, thereby explaining the lack of exacerbated malaria in NOS-deficient mice even though NO may function in protection. However, NOS2- and B cell-deficient mice, which cannot use Ab-mediated immunity, suppress their parasitemia with a similar time course as B cell-deficient controls. C57BL/6 mice treated with *Propionibacterium acnes* to elicit high levels of macrophage-derived NO have a similar time course of *P. chabaudi* parasitemia as *P. acnes*-treated NOS2$^{0/0}$ mice, which do not produce NO; this indicates that NO is not sufficient for parasite killing. Collectively, these results indicate that NO is not necessary or sufficient to resolve *P. chabaudi* malaria. *The Journal of Immunology,* 2000, 165: 3317–3323.

Malaria is the second leading cause of morbidity and mortality due to a single infectious agent. It is a re-emerging disease due to increasing drug resistance by the malarial parasite and insecticide resistance by the mosquito vector. The bloodstage of malaria, which is the focus of our study, causes the signs and symptoms of malaria as well as the pathology. Most individuals (~99%) control bloodstage malaria, suggesting that components of the immune system kill replicating parasites in the blood. However, the precise mechanisms by which bloodstage parasites are killed remain to be determined. We therefore tested whether NO represents an important defense mechanism against bloodstage malaria.

NO has potent biological activity, including maintaining vascular physiology, modulating inflammatory responses, and functioning in host defense against micro-organisms (1–3). This free radical has a biological half-life of seconds under normal physiologic oxygen tension. NO diffuses freely across membranes to mediate its effects. NO plays a vital role in the suppression of the acute parasitemia in experimental *Leishmania major* infections (4) as well in the prevention of reactivation of chronic low grade infection (5).

There are three different isoforms of the NO synthase (NOS)[3] enzyme that produces NO from the terminal guanidino nitrogen of arginine (1). Neuronal NOS (NOS1) is expressed primarily in the brain (6). Inflammatory NOS (NOS2) is expressed by cells of the immune systems, primarily macrophages and neutrophils, and this isoform plays a major role in anti-microbial defense (7). T cells reportedly also produce NO (8). Endothelial NOS (NOS3) is expressed by vascular endothelial cells (9). There are two isoforms of NOS that may contribute to NO production during bloodstage malaria, namely inflammatory NOS and endothelial NOS. Bloodstage malaria in mice and humans results in marked activation of endothelial cells, which, in turn, could contribute to NO production. Thus, endothelial and inflammatory NOS may contribute significantly to NO production during malaria.

The different NOS isoforms have different means of causing the enzyme to produce NO. Neuronal and endothelial NOS are expressed constitutively. Calcium binding to calmodulin within the neuronal or endothelial NOS enzyme complex results in activation of the complex and the production of NO. Inflammatory NOS is normally expressed at low basal levels (10, 11). In contrast to neuronal and endothelial NOS, this enzyme is not regulated by calcium-calmodulin binding and is always active. The levels of expression of inflammatory NOS determine the production of NO by immune cells. Treatment of mice with *Propionibacterium acnes* (formerly called *Corynebacterium parvum*) leads to high levels of NO in the serum of treated rodents, but the animals do not die (12, 13). Serum NO levels peak at about 50-fold and remain at least 10-fold greater than those in untreated controls for >1 wk (12, 13). The ability of *P. acnes* to activate macrophages to produce high levels of NO production allows in vivo testing to determine whether NO can kill replicating malarial parasites.

Unlike the clear role for NO in the resolution of *Leishmania major* and liverstage malaria, the role of NO in the clearance of bloodstage malaria is less defined. Several groups report a relation between protection against bloodstage malaria and NO levels in serum. Favre and colleagues (14) found that IFN-γR$^{0/0}$ mice are more susceptible to *P. chabaudi* infections, and this susceptibility relates to significantly lower serum NOx (NO_2^- plus NO_3^-) levels in IFN-γR$^{0/0}$ mice compared with controls. Stevenson and colleagues (15–17) reported that TNF-α, IFN-γ, and IL-12 are required in vivo to activate serum NO production during *P. chabaudi*

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[3] Abbreviations used in this paper: NOS, NO synthase; AMI, Ab-mediated immunity; CMI, cell-mediated immunity; NOx, NO_2^- plus NO_3^-; AG, aminoguanidine hemisulfate (NOS2 inhibitor); SMT, *S*-methylisothiourea (NOS2 inhibitor).

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malaria and that enhanced NO production relates to protection. The intact spleen is required to resolve bloodstage malaria (18), and splenocytes in C57BL/6 mice up-regulate NOS2 mRNA during *P. chabaudi* malaria. Macrophages from *P. chabaudi*-infected mice produce increased amounts of NO in vitro, suggesting that this cell type is also the source of NO in vivo (19). Collectively, these findings indicate that NO production is induced during bloodstage malaria in splenic macrophages by a pathway that includes IL-12, TNF-α, and IFN-γ. These findings also suggest that NO plays a role in protection against malaria.

Taylor-Robinson and colleagues (20) first reported a definite role for NO in the resolution of *P. chabaudi* malaria. They observed that $CD4^+$ T cell clones of the Th1 type transferred protection to $CD4^+$ T cell-depleted and thymectomized recipient mice, but that treatment of the recipients with NOS2 inhibitors abrogated this protection (20). They concluded that Th1 cells produce NO or activate macrophages to secrete NO that, in turn, kills bloodstage malarial parasites.

In contrast to these results, treatment with the NOS2 inhibitor aminoguanidine (AG) does not alter the time course of *P. chabaudi* parasitemia (16, 21). In experiments performed by Jacobs et al. (16), AG treatment results in some death during ascending *P. chabaudi* parasitemia; Favre et al. (21), however, do not observe this mortality. NOS2-deficient mice resolve *P. chabaudi* (21) and *Plasmodium berghei* XAT (22) parasitemia with a similar time course as C57BL/6 controls, indicating that NO is not required to kill bloodstage parasites.

Results obtained from treatment of CD4-reconstituted SCID mice with a NOS2 inhibitor are in stark contrast with results obtained in NOS2 knockout mice. Differences between contrasting results observed in knockout mice vs treated mice are often explained by the concept of redundancy. Knockout mice with their lifelong deficiency have harnessed other components of the immune system to compensate for the deficiency, whereas treated mice do not have the time to compensate. Compensate means that the depleted protein is replaced by another protein with a similar function. For example, IL-13 may compensate for IL-4 deficiency. Compensation implies that a negative result in knockout mice should not necessarily be interpreted to mean that the missing component has no role. To test whether compensation is an explanation for the contrasting results, we elicited by *P. acnes* treatment 50-fold higher levels of NO than those in uninfected mice during ascending *P. chabaudi* parasitemia and assessed its effect on the infection.

Our results in bloodstage malaria indicate that during an immune response there is cross-talk between different effector arms of the immune response, with each limiting the other's response (23). Thus, if Ab-mediated immunity (AMI) is deficient, then B cells no longer signal to limit cell-mediated immunity (CMI; specifically $\gamma\delta$ T cells), and consequently, CMI is enhanced in bloodstage malaria (23). Conversely, if components of CMI (for example, $\gamma\delta$ T cells) are missing, then $\gamma\delta$ T cells no longer signal to limit the B cell response, and AMI is enhanced (24). We have termed plasticity of the immune response this ability to enhance effector arms of the immune response in the absence of another immune component (24). It is therefore possible that AMI is augmented in the absence of macrophage-derived NO, another component of CMI. If augmentation by AMI for NO occurs, then the parasitemia time course will be similar in NOS-deficient mice and controls even though NO may function in killing malarial parasites. To eliminate this possibility, we examined the role of NO in the resolution of malaria in B cell-deficient mice lacking AMI.

Specifically, we have addressed the following issues to define further the role of NO in resolving bloodstage malaria. First, we examined whether the level of stable breakdown products of NO ($NOx = NO_2^- + NO_3^-$) is markedly increased during descending parasitemia in an attempt to associate NO production with parasite killing by the immune response. Second, we examined which of the two possible isoforms of NO that may contribute to NO production in blood actually produce NO during malaria and whether this isoform is required for clearance of malarial parasites from blood. It is possible that AMI compensates for macrophage-derived NO, much as we had observed earlier for $\gamma\delta$ T cells. In this case, NOS-deficient mice will resolve their malaria with a similar time course as controls even though NO may play a role in protection. Third, we determined the requirement for NO in the resolution of malaria in B cell-deficient mice lacking AMI to rule out possible enhanced AMI replacing the role of NO. Fourth, we assessed whether high levels of NO elicited by *P. acnes* treatment results in killing of parasites to determine whether NO production is sufficient for protection.

Materials and Methods

Mice, parasites, and infection of mice

Female and male C57BL/6 and BALB/c mice between 4–5 wk of age were purchased from The Jackson Laboratory (Bar Harbor, ME) and were infected between 6 and 16 wk of age. B cell-deficient (J_HD) mice, a gift from Dr. Weidanz (University of Wisconsin, Madison, WI), were used to eliminate the possibility of enhanced AMI functioning in place of NO. Breeder $NOS2^{0/0}$ and $NOS3^{0/0}$ mice (C57BL/6 background) were purchased from The Jackson Laboratory. J_HD mice (strain 129 background), which fail to produce Igs due to the targeted deletion of the J_H gene segments in embryonic stem cells, are devoid of surface Ig^+ cells in the periphery because B cell differentiation is blocked at the large $CD43^+$ precursor stage (25). $NOS2^{0/0}$ and $NOS3^{0/0}$ mice lack functional NOS2 and NOS3 genes, respectively (26, 27). J_HD, $NOS2^{0/0}$, and $NOS3^{0/0}$ mice were bred at Louisiana State University Health Sciences Center (Shreveport, LA).

To generate animals lacking both B cells and NOS2, J_HD mice were intercrossed with $NOS2^{0/0}$ mice ($J_HD \times NOS2^{0/0}$). The lack of B cells was verified by obtaining 10 μl of blood from the mouse's tail, lysing the erythrocytes by hypotonic shock, then assessing the percentage of $B220^+$ cells in the blood of the mice by flow cytometry (28, 29). B cell-deficient mice had a similar percentage of leukocytes expressing B220 (<0.2%) as the isotype control, whereas B cell-intact mice had >40%.

PCR analyses for the intact and disrupted NOS2 gene were performed to genotype mice. Briefly, about 50 μl of blood was obtained from each mouse, and the DNA was extracted from the blood with the blood DNA kit from Amersham-Pharmacia (Piscataway, NJ). PCR was performed as described previously (30), except that different primers were used. NOS2 primer 1 (GAGGAGAGAGATCCGATTTAGAGTCTTGG) and NOS2 primer 2 (TGAAGCCATGACCTTTCGCATTAGCATGG) were added to identify an NOS2 gene (26) or neo primer (ACTGCTCGACATTGGGT GGAAACATTCC) plus NOS2 primer 3 (GACAGGTGTGAGCTACCA CATCTGAGTC) to identify the disrupted allele (26). The PCR products were analyzed on an ethidium bromide (Sigma, St. Louis, MO)-stained 1.5% agarose gel (Life Technologies, Gaithersburg, MD). NOS2 knockout mice had no PCR product for NOS2, but did have a disrupted allele product, wild-type mice had a NOS2 PCR product but no disrupted allele product, and heterozygous mice had both PCR products.

LPS-treated mice (20 mg/kg) with no NOS2 PCR product had serum NOx (NO_2^- plus NO_3^-) levels similar to those in untreated controls, whereas mice containing a NOS2 PCR product (both wild-type and heterozygous) had significantly elevated serum NOx levels, confirming that the PCR genotyping of NOS2 gene was correct.

All mice used in our studies were on a defined background except for the J_HDxNOS2 mice, which were a mixture of C57BL/6 and strain 129. To ensure that genetic variability between the different strains did not influence our results, we used B cell-deficient littermates that were NOS2-null homozygotes (as our test group) and NOS2 heterozygotes (as our control group).

Parasites and infection of mice

The malarial parasite *P. chabaudi* adami 556KA, a gift from Dr. William Weidanz, used in these studies was maintained and used as described previously (31). This strain is not lethal in mice with an intact immune system, but results in high levels of unremitting parasitemia in immunodeficient SCID mice (28). Frozen parasite stabilate was injected i.p. into a BALB/c

source mouse, and blood was obtained from the source mouse to generate the inoculum for the experimental animals. Experimental mice were injected on day 0 of infection with 10^6 erythrocytes parasitized with *P. chabaudi*, and the parasitemia was assessed by enumerating between 200 and 1000 erythrocytes in Giemsa-stained thin blood films. In each experiment groups of at least four mice of either sex were used. In experiments using $NOS2^{0/0}$ mice and the time course of NO production, only female mice were used. In all other experiments approximately equal numbers of male and female mice were used in each group to ensure that gender did not influence the results.

Treatment of mice

Mice were injected i.p. with LPS (Sigma) at a dose of 400 μg/mouse in 0.2 ml of saline. Sera were harvested from the LPS-treated mice and analyzed for NOx levels.

P. acnes (Burroughs Welcome) was formalin treated and therefore killed. *P. acnes* were suspended in PBS at a concentration of 7 mg/ml, and 0.3 ml was injected i.p. into each mouse. For a 20-g mouse (the approximate weight of mice used in this study) this represents a dose of about 100 mg/kg. This dose has been used by others (12, 13).

Mice were injected i.p. with 5 μg/mouse of AG or 6.25 μg/mouse of *S*-methylisothiourea (SMT) in 0.1 ml of saline or saline alone. AG and especially SMT at these doses are potent inhibitors of NOS2 (32, 33). These NOS2 inhibitor solutions were prepared fresh daily just before injection.

We injected the treatment i.p in experiments in which repeated injections were required or where a large volume (>0.2 ml) must be provided. Injections i.p. nonspecifically activate cells in the peritoneum, and this nonspecific activation of phagocytes may reduce viability of parasites in the inoculum. Thus, in the NOS2 inhibitor and *P. acnes* treatment experiments, we injected the treatment i.p. and the parasites i.v.

Measurement of serum NOx levels

Mice were anesthetized by i.p. injection of ketamine, then >0.5 ml of blood was obtained by cardiac puncture. The blood was allowed to clot at 4°C for several hours, then the supernatant was removed after centrifugation. The serum nitrate and nitrite levels were determined by first reducing all nitrate to nitrite using nitrate reductase, and then total nitrite was quantified using the Griess reaction (34). All samples in a single experiment were assayed simultaneously to ensure consistency. The serum NOx values for uninfected mice are shown in the figures on day 0 of infection.

Statistical analysis

ANOVA with the StatView program (SAS Institute, Cary, NC) was performed to statistically compare parasitemia and serum NOx levels in the different groups of mice. There were at least four mice in each group. An asterisk in the figures denotes $p < 0.05$.

Results

In *P. chabaudi* malaria, immunologically intact mice suppress their acute infection by about day 14 of infection, then develop chronic malaria that is sterilized by about day 40 of infection. B cell-deficient mice suppress their acute *P. chabaudi* parasitemia with the same time course as C57BL/6 mice, but few B cell-deficient mice sterilize their infections. Instead, B cell-deficient mice have low grade, chronic *P. chabaudi* parasitemia for at least a year (35–37) (our unpublished observations).

NO in serum is significantly enhanced during descending P. chabaudi *parasitemia in C57BL/6 mice, a period when malarial parasites are killed by the immune response*

To determine whether NO production is enhanced in mice during the course of *P. chabaudi* malaria, we injected C57BL/6 mice i.p. with 10^6 erythrocytes parasitized with *P. chabaudi* and analyzed sera from groups of four mice at selected time points during the course of the infection. Serum NOx levels were increased 2- to 3-fold ($p < 0.05$) during peak and descending parasitemia compared with levels in uninfected controls (Fig. 1). The large increase on day 14 compared with levels on days 12 and 10 of *P. chabaudi* infection is probably an experimental artifact, with the average results on day 14 slightly higher and those on days 10 and 12 slightly lower due to experimental variation. In the second experiment similar serum NOx levels were detected on days 12 and 14 of infection, and these were the maximal levels. Upon suppression of the acute *P. chabaudi* infection, the serum NOx levels in C57BL/6 mice on day 20 of infection were similar to those in uninfected controls ($p = 0.5$; Fig. 1).

Inflammatory cell-derived NOS2, but not endothelial-derived NOS3, produces NO during P. chabaudi *malaria, but this NO is not required to suppress* P. chabaudi *malaria in C57BL/6 mice*

To determine whether NO produced at peak parasitemia and during descending parasitemia is actually required for resolution of *P. chabaudi*, we injected $NOS2^{0/0}$ mice with 10^6 erythrocytes parasitized with *P. chabaudi* and analyzed sera from groups of four mice at selected time points during the course of the infection. We selected day 14 of infection because this time point is close to peak serum NOx production; day 20 of infection was also evaluated to verify that no aberrant NO production was occurring in the $NOS2^{0/0}$ mice during chronic malaria.

On day 14 of *P. chabaudi* infection, C57BL/6 mice had significantly ($p < 0.05$) elevated serum NOx levels compared with those in $NOS2^{0/0}$ mice (Fig. 2). The level of serum NOx in $NOS2^{0/0}$ mice was similar on day 14 of infection and on day 20 of infection and in uninfected control $NOS2^{0/0}$ mice. Despite the >5-fold difference in serum NOx in $NOS2^{0/0}$ mice compared with C57BL/6 controls, $NOS2^{0/0}$ mice had a similar time course of *P. chabaudi* parasitemia as C57BL/6 controls (Fig. 2).

To determine whether endothelial NOS3 contributes to NO production and protection against experimental malaria, we injected i.p. $NOS3^{0/0}$ mice and C57BL/6 controls with 10^6 erythrocytes parasitized with *P. chabaudi*. Serum NOx levels in *P. chabaudi*-infected $NOS3^{0/0}$ mice were similar to those in infected C57BL/6 mice on day 14 of infection (Fig. 3). The time course of *P. chabaudi* parasitemia in $NOS3^{0/0}$ mice was similar to that in C57BL/6 controls (Fig. 3). In the second experiment there was a statistically significant ($p < 0.05$) delay in clearance of parasitemia, but this modest delay (3 days) was probably not biologically significant.

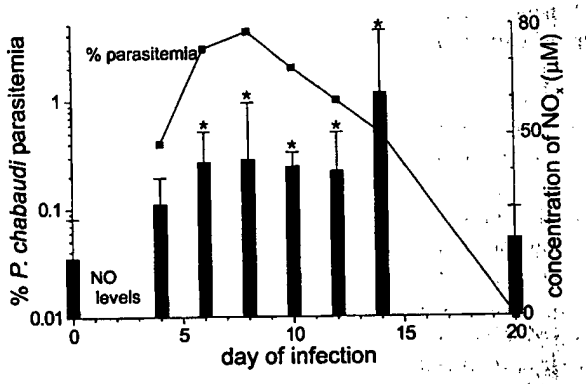

FIGURE 1. Time course of *P. chabaudi* parasitemia and NO production in C57BL/6 mice. C57BL/6 mice were injected i.p. with 10^6 erythrocytes parasitized with *P. chabaudi*, and the parasitemia was assessed (*left axis*, line graph). At each of the selected time points, the level of serum NOx was determined for each mouse (group of four), and the results were averaged (*right axis*, bar chart). The bar on day 0 represents the average serum NOx level in uninfected controls. Three mice of 25 were omitted because their parasitemia was delayed by >2 SD, and their NOx levels differed from their means by >2 SD. The bars on day 0 represent the average serum NOx level in uninfected controls. This experiment was repeated twice with similar results. *, $p < 0.05$ for the comparison with uninfected controls.

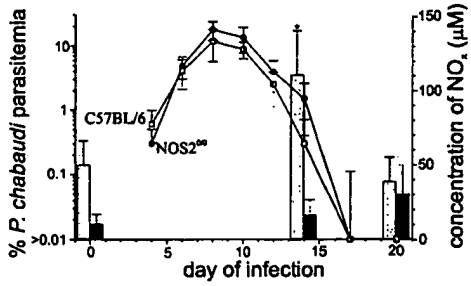

FIGURE 2. Time course of *P. chabaudi* parasitemia and NO production in NOS2-deficient mice and C57BL/6 controls. NOS2-deficient and C57BL/6 mice were injected i.p. with 10^6 erythrocytes parasitized with *P. chabaudi* (*left axis*, line graph). At each of the selected time points, the level of serum NOx was determined for each mouse and averaged (*right axis*, bar chart). ●, NOS2-deficient mice; ▨, C57BL/6 controls. The bars on day 0 represent the average serum NOx level in uninfected controls. This experiment was repeated three times with similar results. *, $p < 0.05$ for the comparison with uninfected controls.

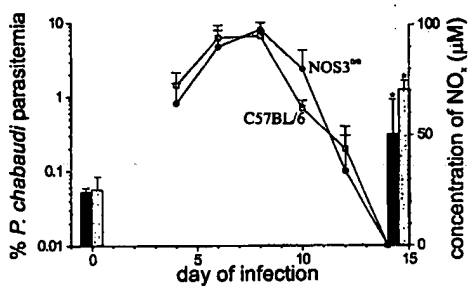

FIGURE 3. Time course of *P. chabaudi* parasitemia and NO production in NOS3-deficient mice and C57BL/6 controls. NOS3-deficient mice and C57BL/6 controls were injected i.p. with 10^6 erythrocytes parasitized with *P. chabaudi* (*left axis*, line graph). At each of the selected time points, the level of serum NOx for each mouse was averaged (*right axis*, bar chart). ●, NOS3-deficient mice; ▨, C57BL/6 controls. This experiment was repeated twice with similar results. *, $p < 0.05$ for the comparison with uninfected controls.

We also evaluated whether NOS3 produces NO in response to a bacterial infection to ascertain whether NOS3, in general, does not contribute to NO production during bloodstage infection. LPS is the component of the bacterial cell wall that induces NO production in vivo. LPS (400 μg/mouse) was injected i.p. into groups of NOS2$^{0/0}$ and NOS3$^{0/0}$ mice and C57BL/6 controls. Serum NOx levels in LPS-treated NOS2$^{0/0}$ mice (48 ± 5 μM) were 1- to 2-fold higher than those in untreated NOS2$^{0/0}$ and untreated C57BL/6 mice (20 ± 13 and 30 ± 10 μM, respectively). In contrast, serum NOx levels in NOS3$^{0/0}$ mice (455 ± 5 μM) were greater than those in LPS-treated C57BL/6 controls (378 ± 11 μM), and both levels were >10-fold higher ($p < 0.05$) than those in untreated C57BL/6 mice (30 ± 10 μM).

NO is not required to suppress acute P. chabaudi *malaria, and plasticity of the immune response is not the explanation for the lack of an effect of NO on parasitemia*

Plasticity of the immune response, with AMI being enhanced in the absence of macrophage-derived NO, a component of CMI, may explain why NOS2-deficient mice do not develop exacerbated *P. chabaudi* malaria. To determine whether NO generated in the absence of AMI is required to suppress acute *P. chabaudi* infections, we injected i.p. 10^6 *P. chabaudi* parasitized erythrocytes into B cell- plus NOS2-deficient mice. B cell- plus NOS2-deficient mice infected with *P. chabaudi* had serum NOx levels on days 10 and 20 of infection similar to those in uninfected controls, whereas infected B cell-deficient mice had significantly ($p < 0.05$) elevated serum NOx levels (Fig. 4). However, B cell-deficient mice lacking NOS2 suppressed their acute infections with a similar time course as B cell-deficient control mice with NOS2 (Fig. 4). B cell- plus NOS2-deficient mice had similar parasitemia during the period of chronic parasitemia compared with control mice lacking B cells but capable of producing NO.

To test whether NOS2 inhibitors have an effect in our hands, we treated B cell-deficient and C57BL/6 mice with inhibitors of NOS2. Groups of five mice were injected i.v. with 10^6 erythrocytes parasitized by *P. chabaudi* then injected i.p. each day with 5 μg/mouse of AG or 6.25 μg/mouse of SMT in 0.1 ml of saline or saline alone. Both AG and especially SMT are potent inhibitors of NOS2 (32, 33). The parasitemia time courses in two separate experiments were similar in treated and control animals, and no animals died during the course of these experiments.

Elevated and sustained NO production induced by treatment with P. acnes *does not mediate enhanced* P. chabaudi *clearance from the circulation*

To determine directly whether NO produced at high levels during ascending parasitemia when the parasites are rapidly replicating can actually kill these bloodstage malarial parasites, we injected C57BL/6 mice i.p. with 0.3 ml of formalin-killed *P. acnes* and then injected these animals i.v. with 10^6 *P. chabaudi*-parasitized erythrocytes 4 days later. This time sequence was chosen because formalin-killed *P. acnes* produced increased levels of serum NOx in C57BL/6 mice on day 4 of treatment (191 ± 8 μM) compared with those in PBS-treated controls (31 ± 4 μM). Serum NOx levels in *P. acnes*-treated C57BL/6 mice peaked on day 8 of treatment (1,069 ± 41 μM), then this high level of macrophage-derived NO declined gradually over a 1-wk period (on day 12, 937 ± 211 μM; on day 16 of treatment, 377 ± 339 μM) (12, 13). Having high levels of NO present at the time of infection gives NO the greatest chance to kill malarial parasites and influence the parasitemia time course. At this time, there is the least likelihood that other molecules induced by infection (such as superoxide) quench NO. Moreover, *P. chabaudi* parasitemia peaks on day 8 of infection; *P. chabaudi* parasites in the *P. acnes*-treated mice therefore are replicating in the presence of continuous high level secretion of NO. We assessed serum NOx levels on day 10 of infection (day 14 of treatment) to verify that significant levels of NO were produced.

Infection control C57BL/6 and NOS2$^{0/0}$ mice injected with PBS at the same time as the test group was treated with *P. acnes* had a similar time course of *P. chabaudi* parasitemia, with peak parasitemia >10% (Fig. 5*A*). Treatment of C57BL/6 and NOS2$^{0/0}$ mice with *P. acnes* significantly ($p < 0.05$) reduced parasitemia in both groups of mice compared with that in PBS-treated controls (Fig. 5*A*). NOS2$^{0/0}$ mice treated with *P. acnes* had levels of serum NOx on days 8 and 16 of infection similar to those in uninfected, untreated NOS2$^{0/0}$ and C57BL/6 control mice (Fig. 5*B*). In contrast, C57BL/6 treated with *P. acnes* had significantly ($p < 0.05$; ~30-fold on day 8 of infection (day 12 after treatment), 10-fold on day 16 of infection (day 20 after treatment)) increased levels of serum NOx compared with those in NOS2$^{0/0}$ mice (Fig. 5*B*). However, the parasitemia was similar in NOS2$^{0/0}$ mice treated with *P. acnes* and in *P. acnes*-treated C57BL/6 controls (Fig. 5*B*). Similar results were obtained in a replicate experiment.

We performed the identical experimental procedure in B cell-plus NOS2-deficient mice and B cell-deficient controls, and the

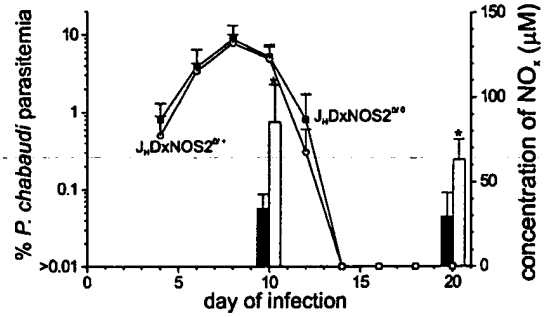

FIGURE 4. Time course of *P. chabaudi* parasitemia and NO production in B cell- plus NOS2-deficient mice and B cell-deficient ($NOS2^{0/+}$) controls. Mice genotyped for B cell production by flow cytometry and NOS2 by PCR were injected i.p. with 10^6 *P. chabaudi*-parasitized erythrocytes, and the average parasitemia was assessed (*left axis*, line graph). Groups of five mice were sacrificed on days 10 and 20 of infection, and serum NOx levels were measured using the Griess reaction.). ■, B cell plus NOS2-deficient mice; ○, B cell-deficient ($NOS2^{0/+}$) controls. This experiment was repeated twice with similar results. *, $p < 0.05$ for the comparison with uninfected controls.

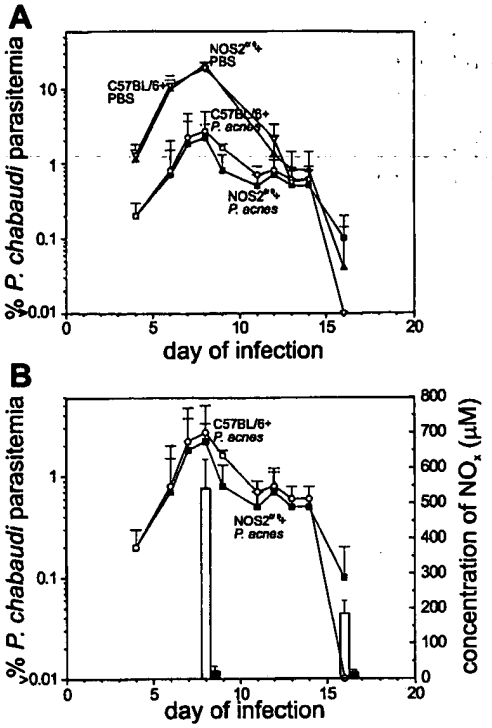

FIGURE 5. *A*, Time course of *P. chabaudi* parasitemia in NOS2-deficient and C57BL/6 mice treated with either *P. acnes* or PBS. *B*, Time course of *P. chabaudi* parasitemia and NO production in NOS2-deficient mice and C57BL/6 controls infected with *P. chabaudi* and treated with *P. acnes*. This experiment was performed twice. Two identical experiments were performed using B cell- plus NOS2-deficient mice and B cell-deficient controls. Five NOS2-deficient or C57BL/6 mice were analyzed for NO production on day 20 of *P. chabaudi* infection, and four mice of each type were analyzed on day 10 of infection. ■, NOS2-deficient mice; ○, C57BL/6 controls treated with *P. acnes*. There was no statistical difference in parasitemia between the two groups at any time point.

results were similar to those described above for B cell-intact mice. On days 10 and 16 of *P. chabaudi* infection, B cell-deficient plus NOS2-heterozygous mice treated with *P. acnes* had significantly ($p < 0.05$) elevated levels of serum NOx compared with B cell- plus NOS2-deficient mice treated with *P. acnes*, yet the parasitemia in the two groups of mice was similar.

Discussion

The role of NO in the clearance of bloodstage malarial parasites remains uncertain. We therefore performed studies to determine whether 1) NO is produced during bloodstage *P. chabaudi* malaria; 2) the lack of the ability to synthesize NO affects the course of *P. chabaudi* malaria; 3) redundancy explains why NOS2-deficient mice resolve their *P. chabaudi* parasitemia with a similar time course as controls; and 4) overproduction of NO kills malarial parasites and consequently alters the course of malaria.

Our observation that serum NOx levels are 2- to 3-fold ($p < 0.05$) elevated during descending parasitemia indicates that NO is present when the immune response is killing parasites. In contrast, Taylor-Robinson et al. (20) have reported that serum NOx was elevated only on a single day during the period of descending parasitemia (~8 days) and that NO was required to suppress *P. chabaudi* parasitemia. How a labile molecule such as NO can be required for suppression of infection yet kill parasites before it is detectable and continue killing beyond the time when it is inactivated was not addressed by these investigators.

Because NO is present during the period when the immune system is killing malarial parasites, we next examined the time course of parasitemia in gene-targeted knockout mice deficient in NO production. We examined mice that were deficient in the two isoforms that may contribute to NO production in the blood, namely, endothelial and inflammatory NOS. Serum NOx levels were elevated on day 14 of *P. chabaudi* infection in $NOS3^{0/0}$ and C57BL/6 mice, but not in the infected $NOS2^{0/0}$ mice, indicating that the NOS2 isoform is responsible for the majority of NO produced during malaria. Our observation that $NOS2^{0/0}$ and $NOS3^{0/0}$ mice resolve their *P. chabaudi* infections with a similar time course as controls together with the studies by Favre et al. (21) and Yoneto et al. (22) indicate that NO is not required for the resolution of acute malaria. *P. chabaudi* parasitemia did not recrudress during chronic malaria in $NOS2^{0/0}$, $NOS3^{0/0}$, and C57BL/6 mice, indicating that NO is not required, as it is in *L. major* infections (5), to maintain protection during the chronic phase of the infection. The parasitemia is also similar in the presence and the absence of NOS2 in B cell-deficient mice with chronic low grade parasitemia.

The ability of NOS isoform-deficient mice to produce serum NO in response to bacterial LPS is similar to the response to *P. chabaudi*. When we injected i.p. NOS-deficient mice with LPS to induce NO production in the blood, the $NOS3^{0/0}$ and C57BL/6 controls had elevated serum NOx levels, but the $NOS2^{0/0}$ mice did not. These data support the concept that NOS2 enzyme functions to produce anti-microbial NO in response to infection, whereas NOS3 produces NO to maintain vascular tone.

One study with pharmacological inhibitors of NOS2 showed marked effects of the inhibitor AG on the time course of *P. chabaudi* parasitemia (20). In contrast, several other studies do not report an effect of the same NOS2 inhibitor on parasitemia (15, 16, 38). Jacobs et al. (16) observe some mortality during ascending *P. chabaudi* parasitemia, whereas Favre et al. (21) do not. We also observed that B cell-intact and B cell-deficient mice treated with inhibitors of NOS2 (aminoguanidine or SMT) resolved *P. chabaudi* malaria with similar time courses as saline-treated controls without mortality. The explanation for the differing results may lie in malarial strain variations, with the strain used by Jacobs et al. (16) being more virulent than that used by Favre et al. (21) and us. The preponderance of evidence obtained from both NOS-deficient and pharmacological inhibitors of NOS2 indicates that NO is not required for suppression of *P. chabaudi* or maintenance of the chronic malaria.

As discussed in detail in the introduction, it is possible that plasticity of the immune response, with enhanced AMI functioning instead of NO, explains the similar time courses of *P. chabaudi* parasitemia in knockout and treated mice compared with controls. However, our finding that mice lacking both B cells and NOS2 resolve their *P. chabaudi* parasitemia with similar time courses as B cell-deficient controls with NOS2 indicates that plasticity by AMI does not explain why NOS-deficient mice do not have exacerbated *P. chabaudi* malaria.

Rockett et al. reported that high levels of NO are toxic to *Plasmodium falciparum* in vitro (38). The lower the oxygen tension, the greater the efficacy of NO against *P. falciparum* in vitro, suggesting that superoxides are scavenging NO (39). In addition, the lack of exacerbation of *P. chabaudi* malaria in the absence of NO does not directly address whether NO can be induced to function in killing malarial parasites. A threshold of NO may be needed for NO to kill malarial parasites. Alternatively, other molecules, such as superoxide, may inactivate NO in vivo during experimental malaria. We therefore examined whether the production of high levels of NO production at the time of infection and during ascending parasitemia would influence the course of *P. chabaudi* parasitemia. We selected treatment with formalin-killed *P. acnes* to induce NO production because 1) *P. acnes* is known to induce nonspecific protection against malaria; 2) it does not produce toxic byproducts like other NO generators; 3) the enhanced NO production occurs for at least 1 wk after a single treatment; and 4) the NO is biologically active (12). *P. acnes* treatment induced increased serum NOx levels by day 4 of treatment with a peak serum NOx on day 8 of treatment (>10-fold higher in *P. acnes*-treated C57BL/6 mice (1069 μM) than in *P. chabaudi*-infected C57BL/6 mice (61 μM); ~50-fold higher in *P. acnes*-treated C57BL/6 mice (1069 μM) than in *P. chabaudi*-infected NOS2-deficient mice (22 μM)). Our observation that *P. acnes*-treated mice with markedly enhanced levels of NO suppress their *P. chabaudi* parasitemia with a similar time course as treated mice lacking NOS2 (with baseline NO) indicates that serum NO is not sufficient to prevent malarial parasites from replicating in blood.

Allison and colleagues as well as Nussenzweig reported decades ago that treatment of mice with *P. acnes* results in nonspecific protection against bloodstage malaria and activation of macrophages (40–42). Several groups report that liver macrophages are the source of NO following *P. acnes* treatment (12, 13). In addition, splenic macrophages are the source of NO during *P. chabaudi* malaria (19). Treatment of mice with *P. acnes* in our experiments results in marked protection against bloodstage *P. chabaudi* malaria and high levels of serum NOx. This protection, as detailed above, is attributable to a mechanism(s) other than NO.

Despite increased levels of NO detected during descending parasitemia, a period when parasites are killed, our results indicate that NO is not required to resolve *P. chabaudi* malaria even after taking into account the possibility of enhanced AMI. The NO produced during *P. chabaudi* malaria is due to inflammatory NOS rather than endothelial NOS. These studies also illustrate the danger of extrapolating function from the enhanced production of a compound. The observation of increased NO during descending parasitemia would lead to an erroneous result in this instance.

Acknowledgments

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Prolonged Th1-like response generated by a *Plasmodium yoelii*-specific T cell clone allows complete clearance of infection in reconstituted mice

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SUMMARY

In the present study, we report the ability of in vitro *cultured CD4$^+$ T cells, generated following immunization with dead blood stage* P. yoelii *parasites, to mediate protection against homologous challenge infection in reconstituted nude mice.* P. yoelii*-specific T cell line cells produced IFN-γ after* in vitro *stimulation with specific antigen, and were protective when adoptively transferred into athymic nude mice. Following transfer of* P. yoelii*-specific T cell lines into nude and SCID mice, elevated levels of nitric oxide (NO) were detected during the first week of infection at a time when parasitaemias were suppressed. However,* in vivo *blocking of NO production through administration of L-NMMA, an inhibitor of NO synthase, increased mortality, but did not alter the course of primary parasitaemia in* P. yoelii*-specific T cell line-reconstituted nude mice. In addition, a* P. yoelii*-specific CD4$^+$ T cell clone, which produced IFN-γ* in vitro*, afforded sterile protection via mechanisms other than NO. By ELISA, antibodies were undetectable on all but one day (day 79) post T cell clone transfer and parasite challenge, where very low levels of antibodies were detected, with some evidence of recognition of malaria proteins by Western blot. Collectively, our data suggest that T cell effector functions, independent of NO production and in the absence of high levels of parasite-specific antibodies, can contribute to sterile immunity to* P. yoelii.

Keywords Plasmodium yoelii, *T cells, protective immunity, nitric oxide*

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INTRODUCTION

Although there is considerable evidence that both antibodies and T cells play a major role in the effector mechanisms of the immune response to the erythrocytic stages of malaria (reviewed by Weidanz & Long 1988), their relative contributions remain unclear. The importance of CD4$^+$ T cells in immunity has been demonstrated in several murine malaria models by *in vivo* depletion of different T cell subsets (Süss *et al.* 1988, Kumar *et al.* 1989, Vinetz *et al.* 1990, Podoba & Stevenson 1991) and adoptive transfer of unfractionated or enriched immune splenic lymphocytes (Jayawardena *et al.* 1982, Cavacini, Long & Weidanz 1986, Kumar *et al.* 1989, Vinetz *et al.* 1990). For *P. chabaudi*, adoptive transfer of protection in immunodeficient mice by CD4$^+$ T cell lines and clones provided further evidence for a role of CD4$^+$ T cells in immunity (Brake, Weidanz & Long 1986, 1988, Taylor-Robinson & Phillips 1993, 1994). Experiments performed in SCID mice, however, demonstrated that although enriched splenic CD4$^+$ T cells were able to control primary *P. chabaudi* infections, they were insufficient to completely eliminate parasites, indicating a need for B cells (and specific antibodies) for parasite clearance (Meding & Langhorne 1991). In addition, B cell deficient mice controlled *P. c. chabaudi*, *P. c. adami* and *P. vinckei* infections to subpatent levels, but did not eliminate parasites completely (Grun & Weidanz 1983, Cavacini, Parke & Weidanz 1990). In contrast, B cell deficient mice were unable to control *P. yoelii* infections (Roberts & Weidanz 1979, van der Heyde *et al.* 1994). However, following drug cure, these mice were capable of resisting a challenge infection with homologous parasites indicating that mechanisms of resistance to reinfection were mediated by antibody-independent mechanisms (Grun & Weidanz, 1983, Roberts & Weidanz 1979). Thus, the mechanism of resistance to acute infection differs depending on the infecting rodent malaria species, with control of

P. yoelii and *P. chabaudi/P. vinckei* infections dependent on antibody-dependent and antibody-independent mechanisms respectively.

The $CD4^+$ T cell effector mechanisms involved in resolution of asexual erythrocytic stages of malaria are not completely understood. The separation of $CD4^+$ T cell into Th1 and Th2 subsets, based on the secretion of cytokines following antigenic stimulation, has greatly facilitated our understanding of the immune mechanisms expressed during infection. Th1 cells produce IFN-γ, IL-2, and TNF-β and mediate delayed type hypersensitivity responses while Th2 cells produce IL-4, IL-5, IL-6, and IL-10, and provide B cell help for antibody production (Mosmann & Coffman 1989). Thus, for *P. c. chabaudi* infections, control of primary parasitaemia was associated with a preponderance of Th1 cells, whereas Th2 cells dominated the resolving phase of infection (Langhorne *et al.* 1989). Furthermore, Th1 and Th2-like clones derived from mice during primary *P. c. chabaudi* infection and after reinfection respectively, adoptively protected $CD4^+$ T cell-depleted mice following *P. c. chabaudi* challenge (Taylor-Robinson & Phillips 1994). In one study, the fall in parasitaemia mediated by the Th1-like clone was associated with a sharp peak in serum nitrate and immunity was impaired by treatment of mice with an inhibitor of inducible nitric oxide synthase (iNOS), L-NMMA (Taylor-Robinson *et al.* 1993). Thus a model for malaria immunity has developed in which the initial phase of the immune response involves activation of an inflammatory T cell response which can control, but cannot eliminate, parasites through a nitric oxide-dependent pathway. Thereafter, final clearance of parasites is dependent upon antibodies, probably specific for agglutinating extracellular merozoites or infected red cells or blocking surface receptors involved in erythrocyte invasion (Miller, Aikawa & Dvorak 1975, Quinn & Wyler 1979). This model, however, has been developed almost exclusively using *P. c. chabaudi*, and it is not known how accurately is reflects the immune mechanisms to other malaria parasites.

P. chabaudi-specific T cell clones generated for studies mentioned above were derived from spleen cells taken from mice which developed immunity following infection and spontaneous cure. Clonal populations of T cells have never been tested for their ability to transfer blood stage immunity to any other species of rodent malaria. In order to broaden our understanding of cell mediated immune mechanisms involved in protection against asexual erythrocytic stages of malaria, we studied the *P. yoelii* model of malaria. Our approach has been to investigate whether T cell clones generated from mice immunized with dead crude *P. yoelii* blood stage antigen can protect immunodeficient mice, to determine the requirements of antibodies and nitric oxide in this immunity, and to ultimately identify protective antigens.

MATERIALS AND METHODS

Mice

Male and female BALB/c and syngeneic BALB/c *nu/nu* and SCID mice were obtained from Animal Resources Centre, Perth, Australia. Mice were housed in the animal facility at the Queensland Institute of Medical Research (QIMR) under conventional conditions. All mice ranged in age from six to eight weeks when experiments were initiated.

Malaria parasites

Cloned *P. yoelii* 17X, nonlethal variant (clone 1·1) was obtained as a generous gift from Dr Walter Weiss, Navel Medical Research Institute, Bethesda, USA. Parasite infections were initiated by intravenous (i.v.) inoculation of 10^6 PRBC harvested from donor mice after one passage of cryopreserved stabilate material. Parasitaemias were monitored by microscopic examination of DIFF-QUIK (Laboratory Aids, Narrabean, NSW) stained tail blood films. Infections with this parasite were typically nonlethal in intact BALB/c mice. By comparison, *P. yoelii* infections were uniformly lethal in BALB/c *nu/nu* and SCID mice. During the course of experiments, however, the parasite became progressively more virulent and produced a rapidly lethal malaria in BALB/c mice.

Preparation of crude *P. yoelii* antigen

When the parasitaemia of infected mice was approximately 30 to 40%, blood was collected by cardiac puncture into heparinised tubes. Erythrocytes were washed ($1000 \times g$ for 10 min) and RBC lysed through three cycles of freezing (two h at −70°C) and thawing (30 min at room temperature). This procedure was followed by disruption (twice for 30 s) with an ultrasonic probe at 80 W on ice. An equivalent number of NRBC from naive BALB/c mice was processed similarly and used as control erythrocyte antigen. This preparation was stored at −70°C until used.

Immunization and challenge

BALB/c mice were immunized subcutaneously (s.c.) with crude *P. yoelii* antigen (equivalent to 10^7 PRBC/mouse) in CFA (H37Ra) (Difco Laboratories, Detroit, Michigan, USA), followed by an intraperitoneal (i.p.) boost of the same dose of antigen without adjuvant approximately three weeks later. A control group of mice were immunized and

boosted with NRBC preparation. A group of untreated mice served as infection controls. All mice were challenged i.v. with 10^6 highly virulent *P. yoelii* PRBC two weeks after booster injection

Generation and expansion of antigen-specific T cell lines

P. yoelii blood stage-specific T cells were generated following repeated *in vitro* stimulation and rest of lymph node cells primed *in vivo* with crude *P. yoelii* antigen. Four to five BALB/c mice were immunized s.c. with crude *P. yoelii* antigen in CFA (H37Ra) (equivalent to 10^7 PRBC per mouse). Seven to nine days after immunization, draining inguinal and popliteal lymph node cells were removed, pooled, and single cell suspensions prepared. Cells were suspended at 2×10^6 cells/ml in complete medium consisting of EMEM (Trace) supplemented with 100 U/ml of penicillin (Commonwealth Serum Laboratories (CSL), Melbourne, Australia) 2×10^{-5} M mercaptoethanol (Sigma Chemical Co., St Louis, MO, USA) and 10% FCS and dispensed in 24 well plates (Corning Glass Works Corning, NY, USA) containing *P. yoelii* PRBC (10^6 PRBC/ml). After four days stimulation, viable cells were isolated on Ficoll-Paque (Pharmacia LKB Biotechnology, Uppsala, Sweden), washed, and dispensed in 24 well plates (10^6 cells/well) containing 2×10^6 irradiated (2500 rads) syngeneic spleen cells in the absence of antigen. This stage served as a rest period for T cells. After 14 to 21 days rest, cells were restimulated with *P. yoelii* PRBC (10^6 PRBC/ml) in the presence of 2×10^6 irradiated spleen cells. T cell lines were maintained by repetitive stimulation/rest cycles for up to eight months.

Ovalbumin-specific T cells were generated using the same procedure adopted for generating *P. yoelii*-specific T cell lines. For *in vivo* priming, BALB/c mice were immunized with 100 μg of ovalbumin (Sigma) in CFA (H37Ra). T cells were expanded *in vitro* by stimulation with 100 μg/ml of ovalbumin.

Generation of *P. yoelii*-specific T cell clones

After three *in vitro* stimulation/rest cycles, established *P. yoelii*-specific T cell lines were cloned by limiting dilution at a density of 0·3 cells/well. T cells were added to 96 well flat-bottomed wells (Nunclon™, NUNC, Denmark) containing *P. yoelii* PRBC (10^6/ml), 10^6 APCs, and 10 U/ml rHuIL-2 (Chiron Corporation, Emeryville, CA, USA). After seven days incubation, 100 μl of medium was removed from each well, and replaced with 100 μl fresh complete medium containing 20 U/ml rHuIL-2 and 4×10^5 irradiated spleen cells in the absence of antigen. After seven days, cultures were restimulated with *P. yoelii* PRBC (without additional rHuIL-2) and incubated for an additional seven days until individual colonies were detected. Cell cultures from wells that exhibited positive growth were expanded and maintained in 24 well plates by alternate stimulation and rest cycles as for T cell lines.

Cell surface phenotype

Rested T cell lines were enriched over a Ficoll-Paque gradient at $400 \times g$ for 20 min. A single cell suspension of the T cell line ($2{\cdot}5 \times 10^5$ cells) in 50 μl of FACS buffer (0·1% BSA, 0·05% EDTA, 0·1% azide in PBS) was dispensed in 96 well round-bottomed plates (Nunclon) and incubated, for 45 min on ice, with 50 μl of undiluted MoAb-secreting hybridoma supernatants. Cells were washed with FACS buffer, followed by 30 min incubation with FITC-conjugated goat anti-rat or anti-hamster secondary antibodies (Caltag Laboratories, South San Francisco, CA, USA) used at 1:100 dilution. Cells were washed, fixed with 1% paraformaldehyde in PBS, and data acquisition and analysis performed on a FACScan© flow cytometer (Becton-Dickinson) with the use of the Lysis II© programme.

Supernatants from the following rat and hamster IgG MoAb-secreting hybridomas were used for staining: KT3 (anti-CD3) and H57-597 (anti-TCR$\alpha\beta$) (kindly provided by Dr T. Mandel, Walter and Elisa Hall Institute (WEHI), Melbourne, Australia); GK1·5 (anti-CD4), 53–6·72 (anti-CD8), and RB3·6B2 (anti-B220, B cell marker) purchased from American Type Culture Collection, Rockville, MD, USA; and GL3·1A (anti-TCR$\gamma\delta$) (kindly provided by Dr K.D. Shortman, WEHI, Melbourne, Australia).

Lymphocyte proliferation assay

Lymph node cells

Seven to nine days after immunization with crude *P. yoelii* antigen, single cell suspensions of the draining inguinal and popliteal lymph node cells were prepared in proliferation medium consisting of EMEM supplemented with 2×10^{-5} M mercaptoethanol and 2% normal mouse serum (BALB/c) at a concentration of 4×10^5 cells/well in 96 well flat-bottomed plates (Nunclon) in the presence of varying concentrations of *P. yoelii* PRBC or NRBC. PPD (10 μg/ml) (CSL, Melbourne, Australia) was used as a positive control.

T cell lines and clones

Following 14 to 21 days culture in the absence of antigen, viable T cell lines or clones were isolated by Ficoll-Paque

gradient centrifugation, washed, and resuspended in proliferation medium. T cell lines and clones were plated at $2-5 \times 10^4$ and $1-2 \times 10^4$ cells/100 μl/well respectively in 96 well flat-bottomed plates containing 1×10^6 irradiated spleen cells/100 μl/well. To test T cell specificity, T cells generated against *P. yoelii* antigens were incubated with *P. yoelii* PRBC or NRBC (10^6 cells/ml) or Con-A (5 μg/ml) (Pharmacia). T cell lines generated against ovalbumin were tested for proliferative response against ovalbumin (100 μg/ml), *P. yoelii* PRBC or NRBC (10^6 cells/ml).

All assays were incubated for four days (37°C, 5% CO_2). [^{3}H]thymidine (6·7 Ci/mmol; Du Pont NEN, Boston, MA, USA) (0·5 μCi in 25 μl EMEM) was added to each well for the final 12–18 h of culture. Cells were harvested onto filtre mats (LKB) and incorporated radioactivity was determined in a liquid scintillation counter. Results are expressed as mean c.p.m. values of three to five replicate wells ± 1 SD. Proliferation was considered significant if the mean c.p.m. of test wells was ≥ 3 SD than the mean c.p.m. of appropriate negative control wells.

Cytokine assay

IFN-γ assay

Assays were performed on 48 h supernatants harvested from 96 well proliferation assays that contained $1-5 \times 10^4$ cultured T cells and 10^6 irradiated spleen cells in 200 μl of proliferation medium. Supernatants were stored at −20°C until use. IFN-γ production was assayed by an enzyme-linked immunosorbent assay (ELISA) kit (Intertest-γ™ Mouse Interferon-γ ELISA, Genzyme Corporation, Boston, MA, USA).

TNF-α assay

TNF-α levels in serum samples were measured using an ELISA kit (Mouse TNF-α Minikit™, Endogen, Cambridge, MA, USA).

Adoptive transfer experiments

Rested T cell lines and clones were enriched over Ficoll-Paque, washed and resuspended in PBS for injection. Recipient mice were administered i.v. either primed lymph node cells (*ex vivo*) or antigen-specific T cell lines or clones at concentrations indicated in the text. Control groups received 10^8 syngeneic normal spleen cells, 10^7 ovalbumin-specific T cells, or no cells. Mice were challenged i.v. with 10^6 *P. yoelii* PRBC 4 to 24 h after cell transfer.

In vivo inhibition of NO production

L-NMMA was used to block, *in vivo*, the synthesis of nitric oxide (NO) by inducible NO synthase (iNOS). L-NMMA, a generous gift of Drs I. Clark, W.B. Cowden and K. Rockett (The Australian National University, Canberra, ACT, Australia) was dissolved in PBS immediately before use and injected i.p. at 4–8 mg per dose every 6–8 h (i.e. each mouse received 24 mg L-NMMA per day). Serum samples, collected prior to and subsequently during treatment were assayed for nitrite and nitrate as a measure of NO production.

Measurement of serum nitrite and nitrate

Quantitation of nitrite

Serum nitrite was quantitated colourmetrically after reaction with Griess reagent (Green *et al.* 1982) according to the method of Rockett *et al.*, 1994. Concentrations were determined directly from a linear standard curve between 1 and 1000 μM sodium nitrite.

Quantitation of nitrate

Serum nitrate concentrations were determined by reducing nitrate to nitrite enzymatically using the enzyme nitrate reductase, as described by Rockett *et al.* (1994) with modifications. Test samples were diluted 1:2 in distilled water and 30 μl incubated with 5 μl nitrate reductase (10 U/ml in distilled water) and 20 μl NADPH (1·25 mg/ml in distilled water) (Boehringer Mannheim, Sydney Australia). Concurrently, a series of nitrate standards (1–1000 μM sodium nitrate, Sigma) prepared in pooled NMS at the same dilution as test samples were reduced in the same manner. NMS controls were again included. Nitrite was measured using the Griess reagent as described above. Plasma nitrate was measured by first subtracting the absorbance of the samples without enzyme from absorbance of samples with enzyme and then deriving concentrations from the standard curve.

Measurement of malaria-specific antibodies

ELISA

Briefly, PVC plates (ICN) were coated with 2 μg/well of soluble antigen (PRBC or NRBC) and incubated overnight at 4°C. Plates were washed three times, incubated with blocking buffer for 1 h, washed three times and incubated with sera for 1 h at 37°C. Plates were washed three times and incubated with horseradish peroxidase-conjugated goat anti mouse IgG (The Binding Site, Birmingham, UK) at a 1:3000 dilution for one hour at 37°C. Colour was developed with 2,2′-azino-di-[ethyl-benzthiozoline sulphonate] (Sigma). Optical density was measured at 405 nm.

Immunoblotting

The ability of serum from recipients of *P. yoelii*-specific T

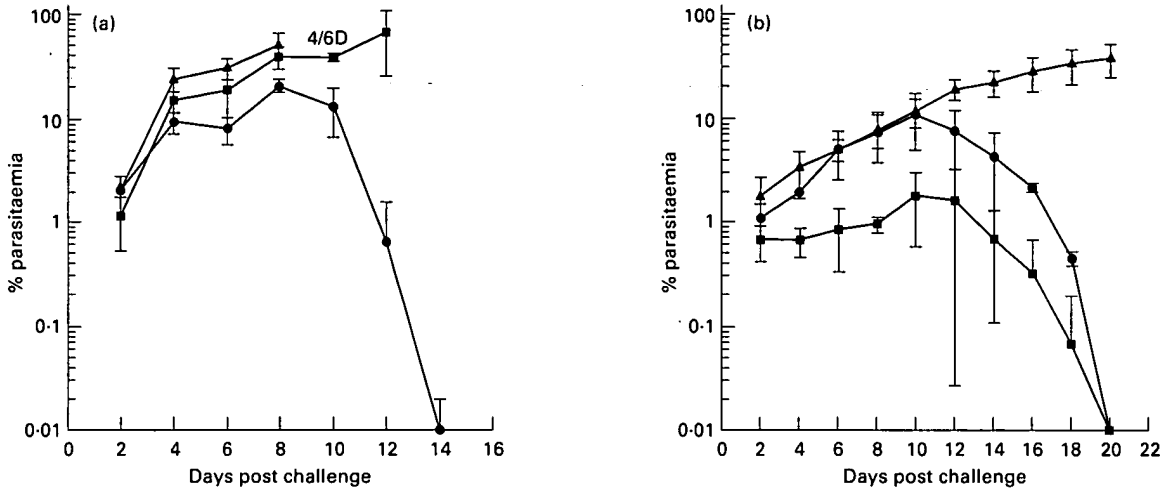


Figure 1 Course of *P. yoelii* infection in immunized and T cell-reconstituted mice. (a) Four BALB/c mice were immunized i.p with *P. yoelii* sonicate in CFA followed by one aqueous boost i.p (●). Six control mice were immunized with an equivalent number of sonicated NRBC in CFA (■). Infection controls (5) were untreated (▲). Mice were challenged i.v. with 10^6 *P. yoelii* PRBC. (b) Nude mice (4–5 per group) were reconstituted with either 10^7 *P. yoelii* primed lymph node cells (●), 10^7 *P. yoelii*-specific T cells (■) or 10^8 naive BALB/c spleen cells (▲). All mice were challenged i.v. with 10^6 *P. yoelii* PRBC 24 h after cell transfer. Results are expressed as mean percent parasitaemias of 4–6 mice ± 1 SD.

cell clones to react with *P. yoelii* PRBC antigens was tested by immunoblotting proteins separated by SDS-PAGE.

Statistical analysis

Significant differences between parasitaemias were determined by applying the one-way analysis of variance (ANOVA).

RESULTS

Immunization with crude *P. yoelii* antigen affords protection against *P. yoelii* infection

BALB/c mice were immunized s.c. with crude *P. yoelii* blood stage antigen in CFA, followed by an i.p. boost of the same dose of antigen without adjuvant approximately three weeks later. A control group of mice were immunized and boosted with NRBC antigen. All mice were challenged i.v. with 10^6 *P. yoelii* parasites two weeks later. Figure 1a shows percent parasitaemias of immunized and control mice. Mice immunized with crude *P. yoelii* antigen developed a mean peak parasitaemia of 19·54% ± 2·43% on day 8 post challenge and all mice resolved their infection by day 14 post challenge. In contrast, naive mice (infection control group) underwent a rapid rise in parasitaemia (range: 28·49%–59·69%) and all mice died by day 10 post challenge. Although mice immunized with NRBC in CFA exhibited lower mean parasitaemias compared to the infection control group, these differences were not statistically significant ($P > 0·1$). Four of six mice died by day 10 post challenge and the remaining two mice died by day 14.

Table 1 Antigen-specific proliferation, IFN-γ production and phenotype of *P. yoelii*-primed lymph node cells and T cell line and clone cells generated against *P. yoelii* antigens

	cpm ± SD			IFN-γ (U/ml)			Phenotype (% positive staining)					
Cells	*Py* prbc	nrbc	mem	*Py* prbc	nrbc	mem	CD3	CD4	CD8	TCRαβ	TCRγδ	B220
Lymph node cells	43 851·7 ± 5785·7	439·5 ± 240·4	452·7 ± 366·6	NT	NT	NT	33·7	29·5	13·0	35·1	0·7	39·7
P. yoelii T cell line	52 644·3 ± 6509·3	757·1 ± 441·6	865·0 ± 322·9	28·0	<0·6	<0·6	98·7	99·4	<0·1	95·2	NT	0·5
Clone G102	4030·0 ± 1162·5	NT	56·5 ± 15·3	47·0	NT	7·7	94·9	96·6	1·2	90·8	NT	0·5

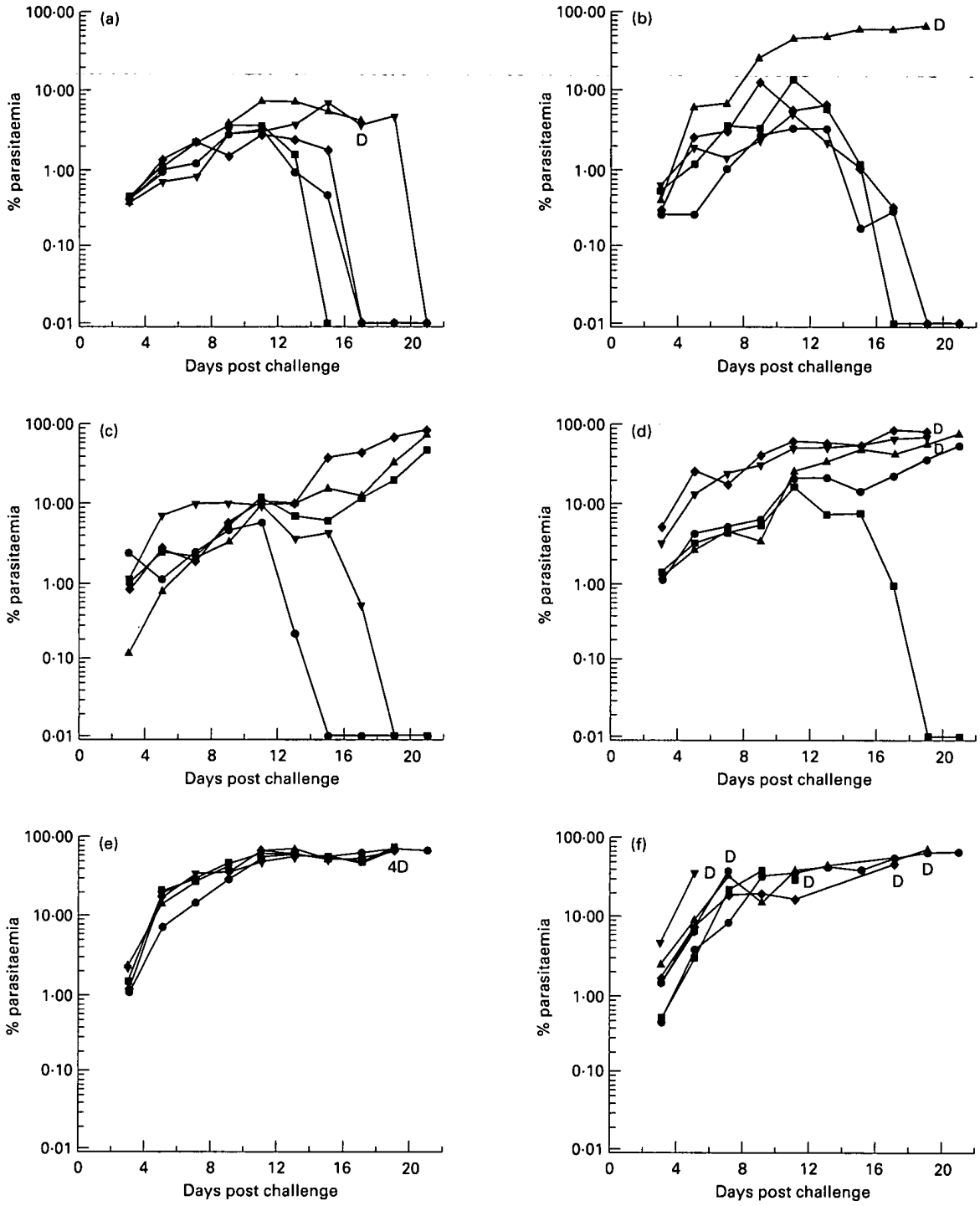


Figure 2 Protective efficacy of *P. yoelii*-specific T cells in reconstituted BALB/c *nu/nu* mice. Recipients (5 per group) were administered i.v. (a) 10^7, (b) 3×10^6, (c) 10^6, (d) 3×10^5, (e) 10^5 rested T cells from *P. yoelii* line. Control nude mice (f) received no cells. All mice were challenged i.v. with 10^6 *P. yoelii* PRBC 24 h after cell transfer. D, Death. Percent parasitaemia is shown for individual mice.

Characterization of T cell response

Table 1 demonstrates the proliferative responses, expression of cell surface markers and IFN-γ production of cell preparations used in adoptive transfer experiments.

Adoptive transfer of *P. yoelii*-primed lymph node cells and T cell lines confers protection in athymic nude mice

Groups of four to six nude mice were administered i.v. 10^7 *P. yoelii*-primed lymph node cells (seven days after immunization) or 10^7 rested *P. yoelii*-specific T cells harvested after three stimulation/rest cycles *in vitro*. A control group received 10^8 syngeneic normal spleen cells. All mice were challenged i.v. with 10^6 *P. yoelii* PRBC 24 h after cell transfer. As demonstrated in Figure 1b, nude mice grafted with *P. yoelii*-primed lymph node cells demonstrated a mean peak parasitaemia of 10·8% ± 5·3% on day 10 post infection and all mice ($n = 4$) resolved their infections by day 20 post challenge. Adoptive transfer of 10^7 T cells from a *P. yoelii*-specific T cell line (Table 1; >99% $CD4^+$) was more effective in suppressing primary parasitaemias than the same number of *P. yoelii*-primed lymph node cells. The mean peak parasitaemia of 1·8% ± 1·1% recorded on day ten post challenge in recipients of the *P. yoelii*-specific T cell line was lower than that observed for mice which received parasite-primed lymph node cells. All mice ($n = 6$) cleared their infections by day 20 post challenge. Control mice receiving normal spleen cells developed escalating infections with a broad peak of parasitaemias ranging from 20% to 50% from day 14 to 20 post challenge.

The efficacy of the *P. yoelii*-specific T cell line in mediating protection was determined by administering five doses of T cells (10^5–10^7) into groups of nude mice. A control group received no cells. All mice were challenged i.v. with 10^6 *P. yoelii* PRBC 24 h after cell transfer. The ability of the T cell line to control parasitaemia was dependent on the number of cells transferred (Figure 2).

NO production in T cell-reconstituted nude mice following challenge infection with *P. yoelii* PRBC

It has previously been reported that immunity to *P. c. chabaudi* mediated by a Th1 clone was associated with the production of NO, and L-NMMA, an inhibitor of iNOS impaired immunity (Taylor-Robinson *et al.* 1993). In the current study, the production of NO as measured by serum nitrite and nitrate was determined following adoptive transfer of *P. yoelii*-specific T cells into nude mice. A group of nude mice ($n = 4$) was administered 5×10^6 *P. yoelii*-specific T cells i.v. per mouse while a control group received no cells. All mice were challenged i.v. with 10^6 *P. yoelii* PRBC 24 h after cell transfer. Serum samples were collected on the days 2, 4, 6, 8, and 10 post challenge and nitrite and nitrate levels measured (Figure 3). Nitrite was undetectable

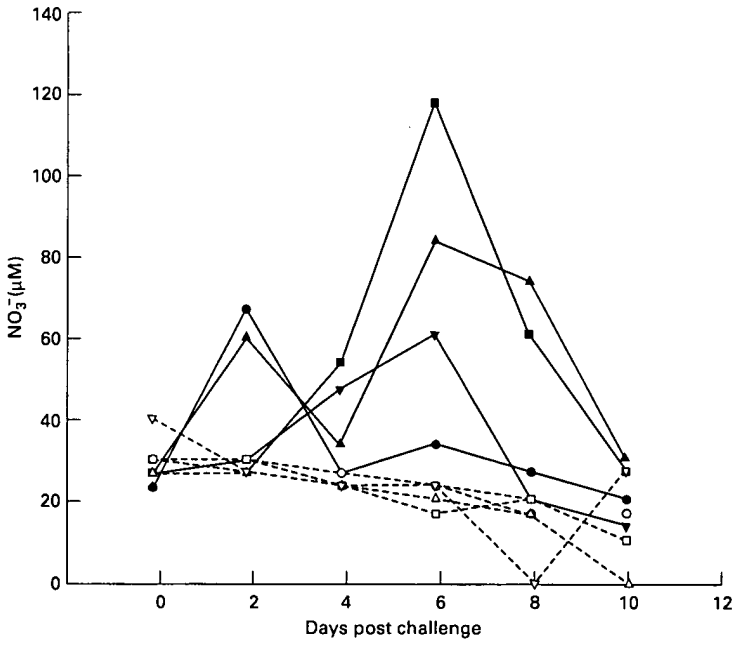


Figure 3 Serum levels of nitrate in individual BALB/c *nu/nu* mice reconstituted with 5×10^6 *P. yoelii*-specific T cells (solid symbols) or no cells (open symbols).

in serum samples of both unreconstituted and T cell-reconstituted nude mice in the presence or absence of parasite challenge (data not shown). In recipients of 5×10^6 *P. yoelii*-specific T cells, nitrate levels were elevated as early as day 2 post challenge and for three mice, reached peak levels on day 6 post challenge. In contrast, no increase in nitrate levels was observed in nude mice receiving no cells.

Effect of *in vivo* inhibition of NO on resolution of infection in reconstituted nude mice

In an effort to understand the role of NO in the control of *P. yoelii* infection *in vivo*, nude mice reconstituted with *P. yoelii*-specific T cells were treated with an inhibitor of NO synthase (L-NMMA) to inhibit the increase in NO production. Two groups of nude mice were administered 5×10^6 T cells *P. yoelii*-specific T cells i.v. Two control groups of nude mice received no cells. All mice were challenged i.v. with 10^6 *P. yoelii* PRBC 24 h after cell transfer. L-NMMA (4 mg per dose) was given i.p. to one group of five T cell-reconstituted mice and five control mice receiving no cells every six h during the ascending phase of primary infection (days 6 to 11). Percent parasitaemia and mortality were monitored during infection. Recipients of *P. yoelii*-specific T cells in the absence of L-NMMA (Figure 4b) demonstrated significantly lower mean parasitaemias from day 8 to 12 post challenge compared to untreated mice receiving no cells (Figure 4d) ($P < 0{\cdot}01$). Reconstitution of nude mice with 5×10^6 *P. yoelii*-specific T cells in the absence of L-NMMA (Figure 4b), although effective in suppressing primary parasitaemias, was not as effective in resolving parasitaemia and preventing mortality, as was shown previously (Figure 2b). One of four mice died by day 15, while the remaining three mice continued to develop ascending parasitaemias. Of the latter, one mouse resolved infection by day 26 post challenge. No significant difference was observed in ascending parasitaemias (from day 2 to 11) between L-NMMA-treated and untreated, T cell-reconstituted nude mice (Figure 4a and b respectively) ($P > 0{\cdot}1$). Differences were noted, however, in terms of early fatality

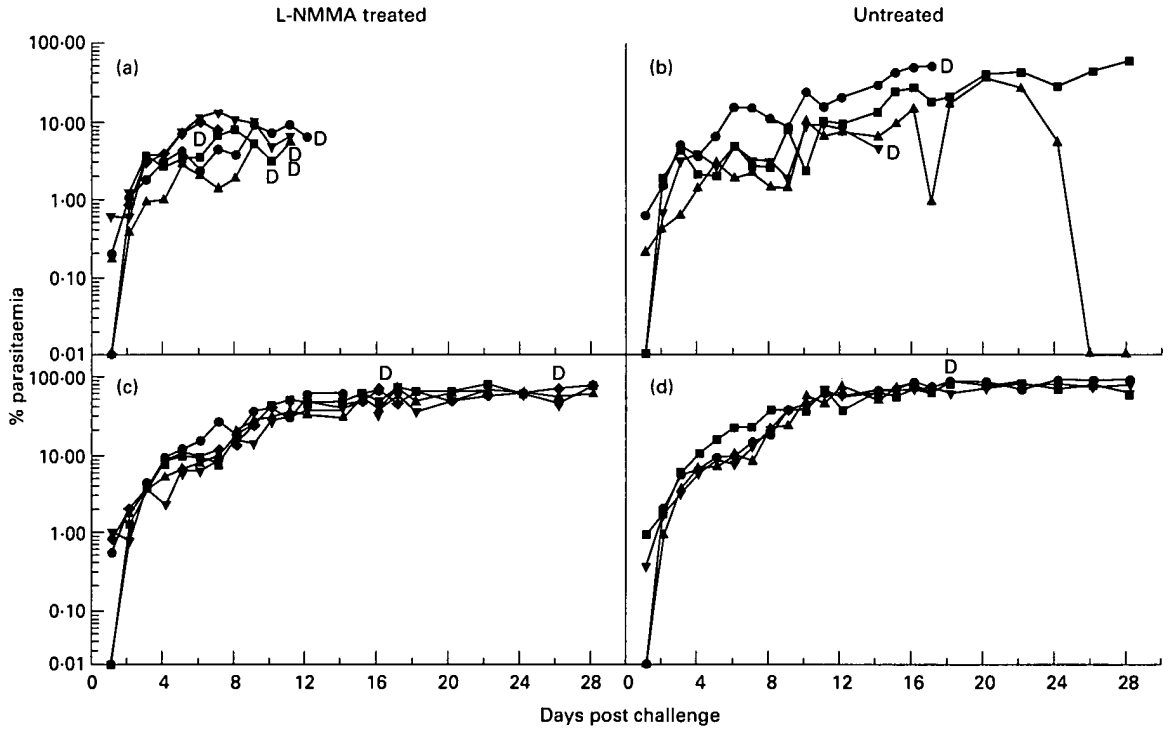


Figure 4 Effect of L-NMMA on course of *P. yoelii* infection in reconstituted BALB/c *nu/nu* mice. (a) Nude mice reconstituted with 5×10^6 *P. yoelii*-specific T cells and treated with L-NMMA (4 mg/dose i.v. every 6 h) on days 6 to 11 post infection. (b) Nude mice reconstituted with 5×10^6 *P. yoelii*-specific T cells in the absence of L-NMMA. (c) Nude mice receiving no cells and treated with L-NMMA. (d) Unreconstituted, untreated nude mice. Recipients (4–5 per group) were infected i.v. with 10^6 *P. yoelii* PRBC 24 h after cell transfer. Serum was collected during L-NMMA treatment and tested for nitrate levels (Materials and Methods). For mice in panel (a), (c), and (d), nitrate was not detected during the ascending phase of parasitaemia (days 6–11). Elevated nitrate levels were detected in three of three untreated, reconstituted controls (b).

observed in T cell-reconstituted, L-NMMA–treated mice, with 100% mortality observed by day 13 post challenge (Figure 4a). NO was detectable (range 25·4–103·4 μM, days 6–8 post challenge) in the serum of three nude mice reconstituted with *P. yoelii*-specific T cells in the absence of L-NMMA. In contrast, T cell-reconstituted nude mice treated with L-NMMA did not have detectable levels of nitrate in their serum (data not shown). Similarly, nitrate was not detected in the serum of untreated controls (Figure 4d). L-NMMA treatment alone had no effect on the course of infection in unreconstituted mice as there was no significant difference in parasitaemias between L-NMMA-treated and untreated controls (Figure 4c and d) throughout the course of infection ($P > 0{\cdot}1$). Serum TNF-α levels were monitored during the ascending parasitaemia in L-NMMA-treated mice since NO inhibition has previously been shown to upregulate the secretion of other cytokines such as TNF and IL-6 (Taio *et al.* 1994). However, TNF was not detected during the period prior to death of nude mice reconstituted with T cells in the presence of L-NMMA (data not shown).

Increased mortality in SCID mice following reconstitution with *P. yoelii*-specific T cells

To determine whether *P. yoelii*-specific T cells could confer protection in the absence of B cells, SCID mice were reconstituted with 10^7, 5×10^6, or 10^6 *P. yoelii* T cell line cells. Control SCID mice received either 10^7 ovalbumin-specific T cells or no cells. Naive BALB/c mice served as infection controls. All mice were challenged i.v. with 10^6 *P. yoelii* PRBC 24 h after cell transfer. Naive SCID mice failed to resolve *P. yoelii* infections and developed high levels of parasitaemia (range: 45% to >80%) before succumbing to infection (Figure 5a). SCID mice reconstituted with 10^7 ovalbumin-specific T cells also failed to control infections and all mice died by day 20 post challenge after experiencing parasitaemia levels in excess of 80% (Figure 5e). In marked contrast, all recipients of 10^7 and 5×10^6 *P. yoelii*-specific T cells (Figure 5b and 5c respectively) and 4 of 5 recipients of 10^6 cells (Figure 5d) died 4 to 6 days post challenge. For these mice, the average parasitaemia at the time of death was 1·25% ($n = 14$). In contrast, the average parasitaemia of mice receiving no cells ($n = 6$) or 10^7 ova-specific T cells ($n = 3$) was higher on days 4 to 6 post challenge (17·27%–23·23% and 13·9%–26·0% respectively). Malaria-specific IgG was not detected in SCID mice which received either 10^7, 5×10^6, or 10^6 *P. yoelii*-specific T cells (Mean OD_{405} prior to death = 0·022 ± 0·012, $n = 15$).

Following reconstitution of SCID mice with 10^7 *P. yoelii*-specific T cells or 10^7 ovalbumin-specific T cells, serum was collected during subsequent infection and nitrite and nitrate levels measured (Table 2). Serum was also collected from BALB/c infection controls and unchallenged recipients of 10^7 *P. yoelii* T cells. As demonstrated previously for T cell-reconstituted nude mice, nitrite was not detected in the serum of infected BALB/c mice or infected SCID mice following reconstitution with *P. yoelii*-specific T cells (data not shown). A modest increase of nitrate levels was observed in infected BALB/c mice from day 0 to 4 post challenge, indicating that NO production is an integral part of the host immune response following infection with *P. yoelii*. Recipients of 10^7 *P. yoelii* T cells (Figure 5b) exhibited a marked increase from baseline levels (day 0) of serum nitrate on day 2 and 4 post challenge (Table 2). While nitrate levels in these mice were not significantly different from controls (SCID mice reconstituted with *P. yoelii* T cells in the absence of parasite challenge) on day 4 post challenge, this may reflect differences in the rate of decrease of nitrate levels in individual mice following peak nitrate production. This increase was not observed in recipients of 10^7 *P. yoelii*-specific T cells in the absence of parasite challenge or in recipients of 10^7 ovalbumin-specific T cells following parasite challenge.

Adoptive transfer of protection with a *P. yoelii*-specific T cell clone

Of seven clones isolated from a *P. yoelii* T cell line, one clone, designated G102, was expanded to numbers sufficient for adoptive transfer. T cell clone G102 was specific for *P. yoelii* PRBC prior to transfer and produced IFN-γ following *in vitro* stimulation with *P. yoelii* PRBC (Table 1). Two BALB/c *nu/nu* mice were reconstituted with 10^6 rested *P. yoelii* clone G102 cells. A control group of nude mice received 5×10^6 T cells from the parent T cell line. All mice were challenged i.v. with 10^6 *P. yoelii* PRBC 24 h after cell transfer. The clone transfer dose of 10^6 cells per mouse was based on previous adoptive transfer experiments in which the same number of *P. chabaudi adami*-responsive T cell clones afforded protection in nude mice (Brake *et al.* 1988). As shown in Figure 6a, ascending parasitaemias in clone G102-reconstituted mice were lower than those exhibited by two recipients of the *P. yoelii* T cell line, with parasitaemias of <5% reached by day 10 post challenge. Recipients of clone G102, however, developed fulminating infections and died by day 29 post challenge.

A second experiment was conducted to determine whether a second injection of clone G102 would allow clearance of *P. yoelii* infection (Figure 6b). Insufficient cell numbers, however, prevented the transfer of 10^6 cloned T cells. Thus, four nude mice were administered i.v. 5×10^5 G102 cells on day 0 and challenged i.v. with 10^6

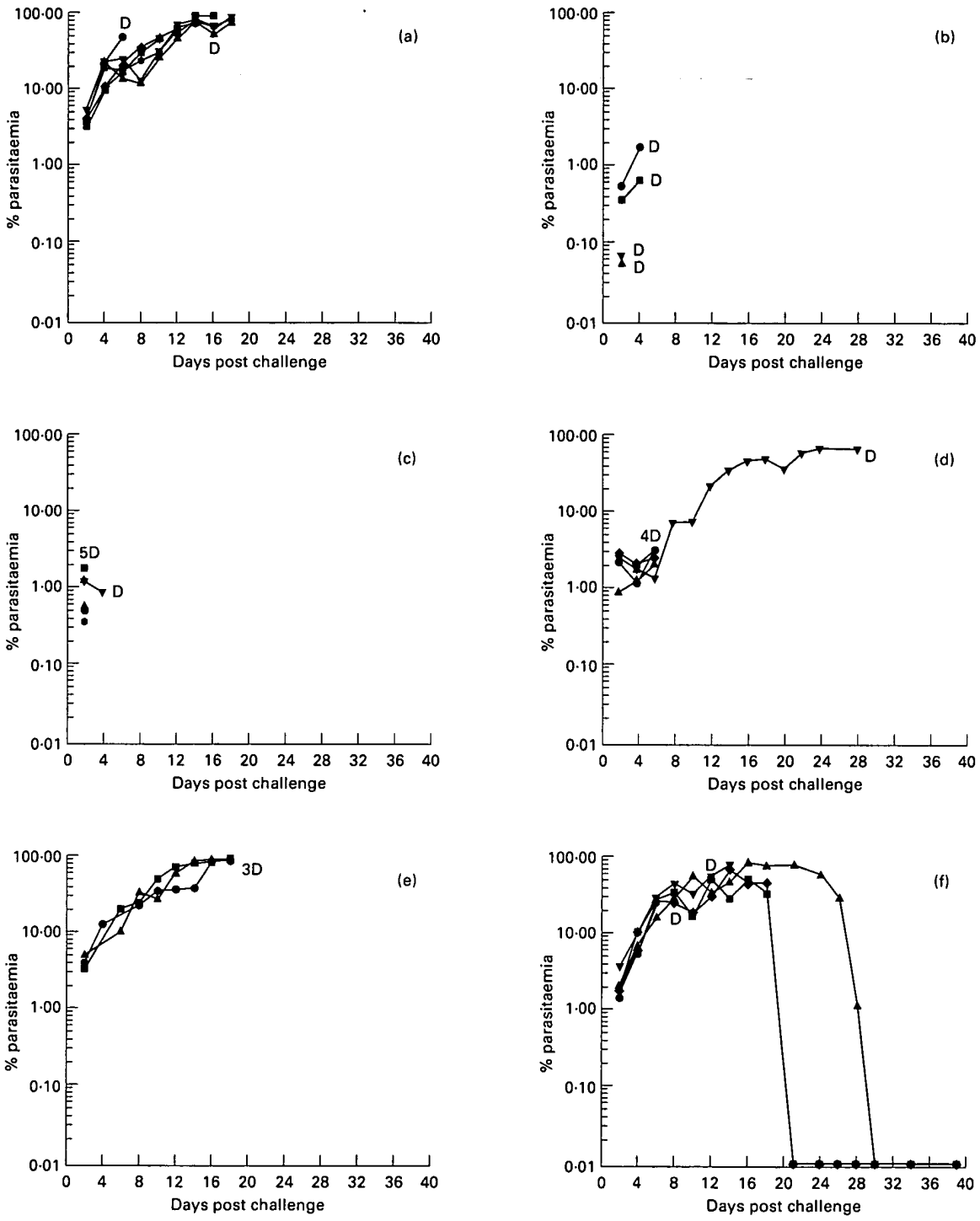


Table 2 Serum nitrate levels in SCID mice infected with *P. yoelii* following reconstitution with *P. yoelii*-specific T cells

	Serum nitrate (μM ± SEM)			
	Days post challenge			
Group	0	2	4	6
SCID infection control	8·7 ± 4·3	15·3 ± 4·9	2·6 ± 4·3	4·7 ± 9·4
SCID + 10^7 *P. yoelii* T cells + challenge	7·0 ± 3·3	484·0 ± 93·4*	716·1 ± 618·3	†
SCID + 10^7 *P. yoelii* T cells (no challenge)	5·0 ± 1·7	17·0 ± 7·7	4·9 ± 6·4	1·0 ± 1·7
SCID + 10^7 Ova T cells + challenge	2·7 ± 1·9	22·7 ± 8·2	6·5 ± 6·0	1·3 ± 1·8
BALB/c infection control	9·6 ± 2·0	28·0 ± 3·2	42·3 ± 5·1	34·4 ± 3·0

* $P < 0{\cdot}04$ *vs* SCID + 10^7 *P. yoelii* T cells (no challenge). †, dead.

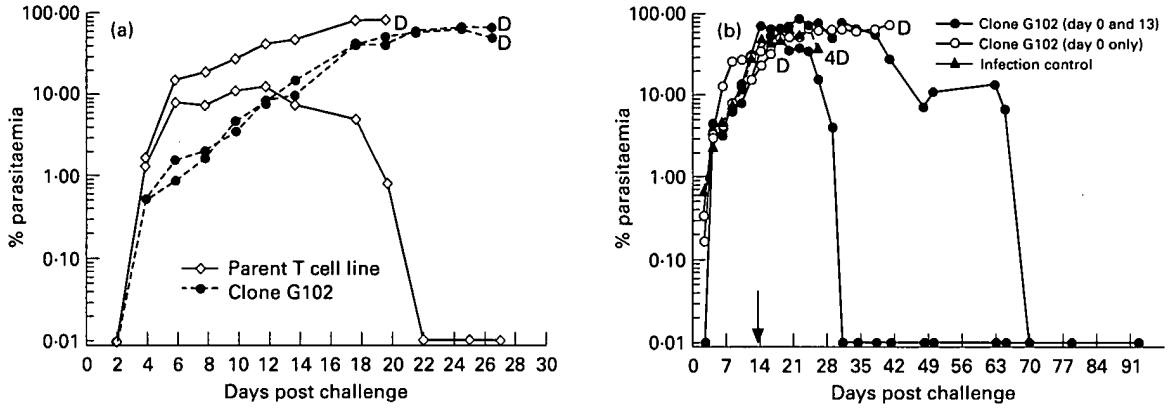
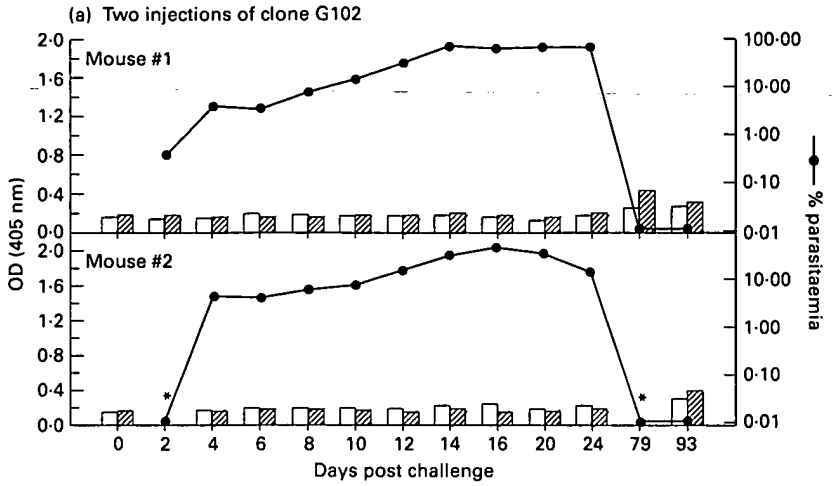
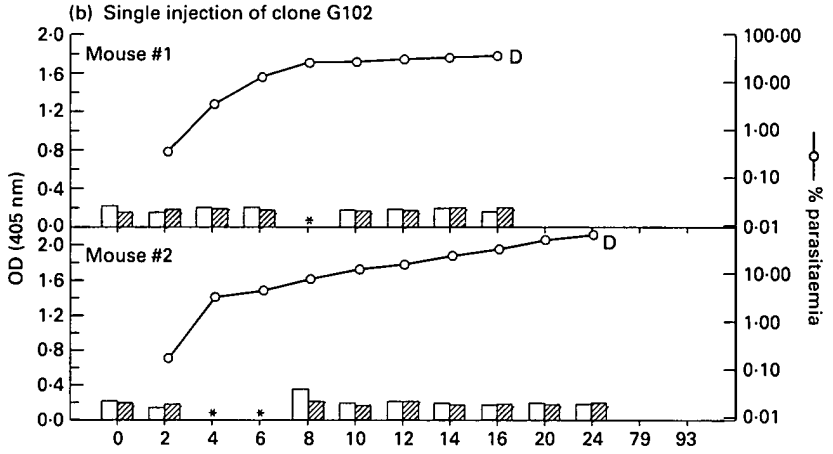


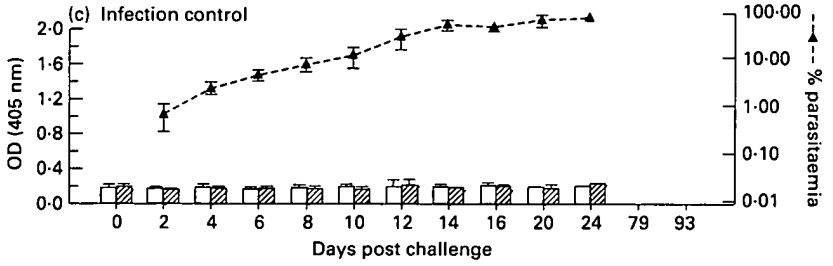
Figure 6 Adoptive transfer of *P. yoelii* specific T cell clones in BALB/c *nu/nu* mice. (a) Recipient mice (two per group) were reconstituted i.v. with 10^6 G102 (●) or 5×10^6 T cells from *P. yoelii*-specific T cell line (◇). All mice were challenged i.v. with 10^6 *P. yoelii* PRBC 24 h after cell transfer. (b) Recipient mice (4) were reconsituted i.v. with 5×10^5 G102 cells. Control mice (▲) received no cells. All mice were challenged i.v. with 10^6 *P. yoelii* PRBC 24 h after cell transfer. On day 13 post challenge, 2 of 4 recipients of clone G102 were administered i.v. a second dose of G102 cells (10^5 per mouse i.v.) (●) and two with PBS (○). Arrow indicates second injection. The course of parasitaemia for recipients of T cell clone G102 and *P. yoelii*-specific T cell line are indicated for individual mice. Results for infection controls (▲) are presented as mean percentage values of four mice. D, Death.

P. yoelii PRBC 4 h after cell transfer. On day 13 post challenge, two of these reconstituted mice were administered 10^5 G102 cells, while the remaining two mice received an injection of PBS. A control group of four nude mice was challenged i.v. with 10^6 *P. yoelii* PRBC on day 0. Infections in unreconstituted, challenged controls were fatal within 29 days. Unlike recipients of 10^6 G102 cells, recipients of 5×10^5 G102 cells were unable to suppress ascending parasitaemias, exhibiting infection kinetics similar to infection controls. Both of these mice succumbed to infection, one by day 18 and the other by day 48 after reaching parasite levels of 34·4% and 69·7% respectively. In contrast, recipients of two doses of clone G102 resolved their infections by day 29 and 70 after parasite levels reached 50·3% and 82·06% respectively. To determine whether sterile immunity was achieved in

Figure 5 Protective efficacy of *P. yoelii*-specific T cells in reconstituted SCID mice. Recipients (3–5 per group) were reconstituted with (b) 10^7, (c), 5×10^6, and (d) 10^6 rested T cells from *P. yoelii* line. Control SCID mice received either 10^7 ovalbumin-specific T cells (e) or no cells (a). BALB/c infection controls were run in parallel to monitor normal course of infection (f). All mice were challenged i.v. with 10^6 *P. yoelii* PRBC 24 h after cell transfer. D, Death. Percent parasitaemia is shown for individual mice.







recovered mice, 250 μl of blood was collected from these mice and inoculated into naive donor BALB/c mice. Examination of blood films taken from donor mice revealed no parasite growth over a 21 day period.

Following challenge infection, nude mice reconstituted with clone G102 (single and repeated dose) were bled on days indicated in Figure 6b and serum nitrate levels measured. T cell clone G102 did not mediate increased nitrate levels during the ascending phase of *P. yoelii* infection (i.e. day 2–24 post challenge) (data not shown). Elevated nitrate levels, however, were detected in recipients of two doses of T cell clone on day 93 post challenge (average NO_3^- of 193·4 μM compared to an average baseline level of 38·3 μM on day 0).

Measurement of malaria-specific IgG in the serum of clone G102-reconstituted mice

Nude mice reconstituted with T cell clone G102 (single or repeated dose) or no cells were bled on days indicated in Figure 7 and the serum analysed for *P. yoelii*-antigen reactive antibodies using ELISA. Insignificant levels of *P. yoelii*-specific IgG was detected in the serum of infection controls (Figure 7c) or recipients of a single dose of clone G102 (Figure 7b) during the course of infection. Similarly, serum samples taken from mice that recovered following two injections of clone G102 did not contain significant levels of parasite-specific IgG, with the exception of low levels of antibodies on day 79 for mouse #1 ($O.D._{PRBC} - O.D._{NRBC} = 0{\cdot}181$ at 1:100 dilution of serum) (Figure 7a).

Western blot analysis was performed to confirm this finding. *P. yoelii* blood stage antigens were subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted with pooled day 79 sera from recovered mice that had received two injections of clone G102. Immune serum (IS) from BALB/c mice that had resolved infections with *P. yoelii* and normal nude serum (NNS) were used as controls. Figure 8 shows the reactivity patterns of pooled sera taken on day 79 from individuals reconstituted twice with clone G102 (A: 1:100 dilution; B: 1:500 dilution). Day 79 serum tested at 1:100 dilution (A, lane 2) recognized several *P. yoelii* blood stage antigens. At 1:500 dilution, however, much of this reactivity was lost (B, lane 2), confirming low antibody titres.

DISCUSSION

Data from the present study established that immunization of BALB/c mice with dead blood stage *P. yoelii* antigen induced a population of IFN-γ secreting, $CD4^+$ T cells which adoptively transferred protection to athymic nude mice. Protection was manifested as either suppression of primary ascending parasitaemias or suppression followed by resolution of infection and was dependent on the number of cells transferred. These results are consistent with previous studies in which a role for $CD4^+$ T cell-mediated immunity against *P. yoelii* infection was demonstrated following adoptive transfer of enriched immune spleen cells prepared from mice immunized via active infection (Jayawardena *et al.* 1982, Vinetz *et al.* 1990). Furthermore, a *P. yoelii*-specific $CD4^+$ T cell clone which produced IFN-γ conferred sterile protection in nude mice. To our knowledge, our data is the first demonstration of protection by T cell clones against blood stage *P. yoelii* parasites.

Studies employing the *P. c. chabaudi* model of malaria have previously demonstrated that during primary parasitaemia in intact mice or following adoptive transfer of a protective Th1 clone into $CD4^+$ T-cell-depleted mice, there was a sharp peak of NO production, as measured by serum nitrate, which coincided with peak parasitaemia (Taylor-Robinson *et al.* 1993, Taylor-Robinson & Phillips 1994). $CD4^+$ T cell depleted mice reconstituted with protective Th2 cells, however, did not exhibit elevated serum nitrate levels, supporting a Th-1-regulation of NO production. In the present study, the transfer of *P. yoelii*-specific T cell line cells into nude mice led to a gradual increase in serum nitrate levels, evident as early as day 2 post challenge and persisting for up to eight days. This was at a time when the *P. yoelii*-specific T cell line was effective in suppressing ascending parasitaemias. To address the role of NO in the resolution of primary *P. yoelii* infection, recipients of *P. yoelii*-specific T cells were treated *in vivo* with L-NMMA, an inhibitor of iNOS, during ascending parasitaemia. L-NMMA treatment did not alter the course of primary *P. yoelii* infection in T cell reconstituted mice but did result in increased mortality at low parasitaemias (ranging from 3 to 7% at the time of death). In contrast, L-NMMA-treatment of $CD4^+$ T cell depleted mice reconstituted with a Th1 clone specific for *P.c. chabaudi*

Figure 7 Measurement of *P. yoelii*-specific IgG during the course of *P. yoelii* infection following reconstitution with two injections of clone G102 in BALB/c *nu/nu* mice. Following infection of BALB/c *nu/nu* mice with 10^6 *P. yoelii* PRBC, serum was collected on the days indicated from (a) recipients of two injections of clone G102 (b) recipients of a single injections of clone G102 or (c) infection controls. Parasite–specific (hatched bars) and NRBC-specific (open bars) IgG antibody levels are shown as optical densities (OD). Asterisks indicate insufficient serum for testing. Percent parasitaemias (indicated by symbols ● and ○) for recipients of clone G102 are indicated for individual mice. Percent parasitaemia for infection controls (▲) are presented as mean percentage values of four mice ± 1 SD.

blood stage parasites resulted in both an increase in peak parasitaemia and mortality (Taylor-Robinson *et al.* 1993). The findings of the present study are difficult to interpret since reconstituted recipients treated with L-NMMA died prior to resolution of infections. The early mortality observed in nude mice reconstituted with *P. yoelii*-specific T cells and treated with L-NMMA could not be attributed to possible toxic effects of L-NMMA since treatment of unreconstituted, infected mice did not result in increased mortality compared to untreated controls. Taio *et al.* (1994) also observed reduced survival rate for endotoxaemic rats treated with an NO synthase inhibitor. It was also noted that pretreatment with NO synthase inhibitor resulted in increased TNF and IL-6 levels. It has been proposed that excessive generation of TNF is linked with the illness and pathology seen in malaria (Clark 1987, Clark, Chaudri & Cowden 1989). However, in the current study TNF was not detected during the period of L-NMMA treatment. Possible reasons for the failure to detect circulating concentrations of TNF include the short half life of soluble TNF or increased serum levels of soluble TNF receptors (Kern *et al.* 1992). Ultimately the use of neutralizing anti-TNF antibodies in conjunction with L-NMMA treatment may determine the role of TNF in mortality. On the other hand, increased mortality in L-NMMA treated mice, together with the inability of NO inhibition to alter ascending *P. yoelii* infection, may reflect a host protective role by NO, as apposed to an anti-parasitic role, as has been proposed by Yap *et al.* (1994).

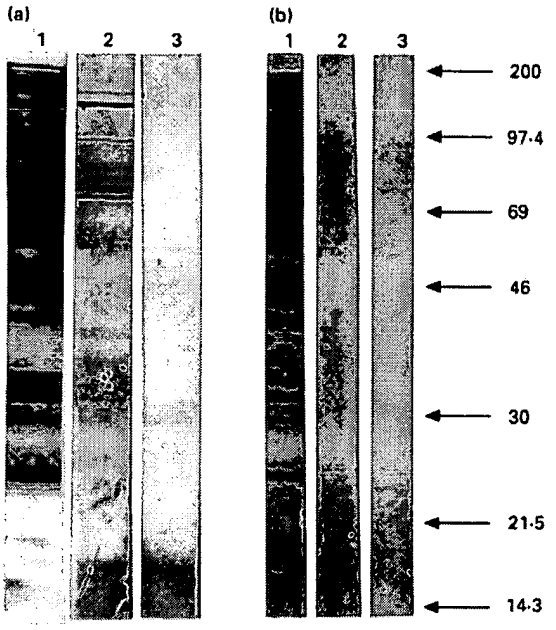


Figure 8 Immunoblot analysis of *P. yoelii* PRBC antigens recognized by serum collected on day 79 from mice reconstituted with clone G102. Pooled sera was tested at (a) 1:100 and (b) 1:500 dilution. Lane 1, immune BALB/c serum (positive control); Lane 2, pooled day 79 sera; Lane 3, normal nude serum.

An interesting finding revealed by adoptive transfer studies was the early mortality observed in SCID mice reconstituted with *P. yoelii*-specific T cells. In these experiments, dramatic increases in serum nitrate (40 to 300 times that observed prior to T cell transfer and infection) were observed in recipients of 10^7 *P. yoelii*-specific T cells. For these mice, the average survival time after challenge infection was three days, despite low levels of parasitaemia prior to death. One possible explanation for this observation may be the excessive production of NO as a result of IFN-γ or TNF production following activation of the *P. yoelii*-specific T cells. B cells have been proposed to indirectly down-regulate Th1 activity through their production of IL-10. IL-10 inhibits cytokine release by Th1 cells and decreases the expression of MHC class II antigen on macrophages (Bogdan, Vodovotz & Nathan 1991, Fiorentino *et al.* 1991). Thus, the absence of regulatory B cells may result in overproduction of IFN-γ and subsequently TNF, resulting in host pathology.

Reconstitution of nude mice with a Th1-like T cell clone specific for *P. yoelii* antigen provided partial protection against *P. yoelii* challenge as evidenced by retarded parasite growth during ascending parasitaemia. Moreover, complete parasite clearance was achieved following administration of a second dose of clone G102 cells approximately two weeks after initial cell transfer. Nude mice reconstituted with T cell clone G102 did not develop antibodies during the primary infection, although low levels of antibodies were detected in the serum of one mouse collected 79 days after challenge when infections were cleared. One explanation for low serum antibody levels might be that antibodies are complexed with parasite antigen and are thus removed from the circulation (Sayles & Wassom 1991). This would be particularly apparent when parasite burden was high, as was the case during primary infection. This explanation, however, does not explain the low antibody levels detected following clearance of infection. That these antibodies alone made a major contribution to resolution of infection was also disputed by passive transfer studies in which administration of 250 μl of day 79 serum in nude mice did not alter the course of infection compared with control mice treated with normal nude serum (data not shown). It is well documented, however, that passive serum transfer studies in this model have a number of inherent problems and must be interpreted with caution (Jayawardena *et al.* 1978). Another explanation for low antibody production during early infection may be explained by observations of Playfair &

De Souza (1979) which suggested that macrophage activation may be responsible for suppression of antibody production.

In conclusion, the current study demonstrated the ability of $CD4^+$ T cell lines and clones specific for *P. yoelii* blood-stage parasites to confer protection in immunodeficient mice. The mechanisms by which parasite levels are controlled or cleared remain speculative. The data would suggest that NO does not play an anti-parasitic role in *P. yoelii* infections. In the absence of high levels of malaria-specific antibodies during the acute primary parasitaemias, we propose that release of IFN-γ by protective T cell lines and clones plays a vital role in activating effector cells such as macrophages, which may exert an anti-parasiticidal effect indirectly by the release of parasite inhibitory factors other than NO, such as TNFα (Stevenson, Tam & Nowotarski 1990), H_2O_2 or O_2^- radicals (Brinkmann *et al.* 1984, Ockenhouse & Shear 1984, Shear *et al.* 1989).

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